

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**ROLE OF ANTIBODIES PRODUCED AGAINST
INNATE IMMUNITY PROTEINS IN THE
PATHOGENESIS OF GASTROENTEROLOGICAL
DISEASES ASSOCIATED WITH CHRONIC (BOWEL)
INFLAMMATION AND THEIR SIGNIFICANCE IN
THE PREDICTION OF DISEASE COURSE**

Nóra Sipeki MD

Supervisor: Mária Papp MD, PhD



**UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL**

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Role of antibodies produced against innate immunity proteins in the pathogenesis of gastroenterological diseases associated with chronic (bowel) inflammation and their significance in the prediction of disease course

By: **Nóra Sipeki MD**

Supervisor: **Mária Papp, MD, PhD**

Kálmán Laki Doctoral School, University of Debrecen

Head of the **Examination Committee**: Prof. György Balla, MD, PhD, DSc, MHAS

Members of the Examination Committee:

Zoltán Szepes, MD, PhD

Prof. Zoltán Prohászka, MD, PhD, DSc

The Examination takes place at the Library of the Department of Pediatrics, Faculty of Medicine, University of Debrecen, 16th of December, 2016. 11:00 am.

Head of the **Defense Committee**: Prof. György Balla, MD, PhD, DSc, MHAS

Reviewers: Zoltán Griger, MD, PhD

Márk Juhász, MD, PhD

Members of the Defense Committee:

Zoltán Szepes, MD, PhD

Prof. Zoltán Prohászka, MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 16th of December, 2016. 13:00 pm.

1. Introduction

Innate immunity considered to be the first line of defense against invading pathogens and potentially harmful agents. This non-selective and non-specific innate immune response cannot induce the development of immune memory, therefore it is not longlasting. However holding up the defense of the organism until activation of adaptive immune system, its significance should not be questioned. The key role of innate immune system in the activation of adaptive immune system has also importance. The most significant humoral (complement system, anti-microbial peptides and different cytokines) and cellular (phagocytes, natural killer and dendritic cells) components are in a “ready-to-act” state independently from the presence of pathogens, being able to act promptly. Recognition of invading pathogens is done by different classes of germ line-encoded pattern recognition receptors (PRR), which monitor the extracellular and intracellular compartments of host cells for signs of microbes. Sequential detection of a pathogen by various PRR in different subcellular compartments is essential and results in activation and a complex interplay of downstream-conserved signaling pathways. PRRs are widely distributed in different forms with various functions all over the human body. They are abundant at the sites of possible entry for pathogenic microorganisms.

PRRs are anchored in innate immune cells as a surface or an intracellular receptors and are involved in signaling, resulting in an inflammatory response and a subsequent cellular activation. The other type of PRRs includes various soluble receptors that move around freely and are considered as functional ancestors of the immunoglobulins (Ig). They act as phagocytic receptors mediating direct non-opsonic uptake of pathogenic microbes and/or their products. On the basis of their function, scavenger receptors (SR), which are cell membrane bounded PRRs also belong to this latter group. These molecules recognize conserved structures designated pathogen-associated molecular patterns (PAMPs) on microbes. Many of these molecules are present in commensals and opportunistic pathogens (MAMPs, microbial-associated molecular patterns). Moreover, PRRs interact not only with exogenous microbial molecules, but also with endogenous structures. Damaged or stressed cells that pose “danger” to self-tissues are recognized through danger (or damage)-associated molecular patterns (DAMPs). A multifaceted interplay of different PRRs results in a complex spectrum of pro- and anti-inflammatory, immunogenic and suppressive responses induced within the host.

Altered expression and function of the PRRs can lead to inappropriate recognition and elimination of pathogens as well as endogenous damaged structures resulting in a defective control of regeneration and inflammation. These altogether can exaggerate the acute and chronic inflammatory processes and alter susceptibility to co-morbidities and can also modify the initial disease course.

Production of antibodies against microbial structures recognized by aforementioned PRRs has been reported in various diseases. The detection of these antibodies from patients' sera is performed via serologic laboratory methods. Antibodies can be divided into subgroups of **anti-microbial** and autoantibodies. Anti-microbial antibodies are formed against surface carbohydrates (**anti-glycans**) or protein antigens of gut microbes. The first and still most relevant anti-glycan antibody to be identified is **ASCA (anti-Saccharomyces cerevisiae antibody)**. Lately anti-OmpC, **anti-OMP**, anti-I2, and anti-CBir1 antibodies were discovered; these are directed against microbial peptides. The targets of autoantibodies are the constituents of various host proteins. Their existence however, might also be related to gut microbes. For several years, the exact mechanisms responsible for the induction of serologic antibodies and formation cite remained unclear. Furthermore, it is debated if these antibodies are bystanders only or whether they actively participate in disease pathogenesis. In the presence of certain functional polymorphism of variant PRR genotypes (MBL deficiency, NOD2 variants) higher antibody production has been observed, which along with disease

specific occurrence of anti-microbial antibodies (disease specific antibody patterns) supports the hypothesis of genetic-based loss of immunotolerance to gut microbiom. The disturbed gut innate immune system than as a consequence may trigger an exaggerated adaptive immune response. According to recent publications, antibodies against certain host proteins (**autoantibodies**) can reflect an altered immune response to gut microflora. Numerous hypotheses were suggested behind the production of **anti-neutrophil cytoplasmic antibodies (ANCA)**. One of them is the phenomenon of molecular mimicry, where a foreign antigen shares sequence or structural similarities with self-antigens. In this case it means that the generation of ANCA is linked to the cross-reaction between certain bacterial proteins and host antigens. Recent findings also support this view, for example it has been reported that the presence of atypical P-ANCAs may reflect an abnormal immune response to intestinal microorganisms. In autoimmune liver disorders, atypical P-ANCA is directed against human β -tubulin isotype-5 (TBB-5) and cross-react with the bacterial protein FtsZ. This is the consequence of that TBB-5 shares an extraordinarily high structural homology with this microbial cell division protein, which is present in almost all bacteria of the intestinal microflora. Occurrence of P-ANCAs has also been considered as a sign of immunological response to enteric bacterial antigens in other diseases. Moreover, there is lack of P-ANCAs in animal models in a germ-free environment supporting also this hypothesis.

Recently identified glycoprotein targets of exocrine **pancreas autoantibodies (PAB) (GP2 and CUZD1)** belong to protein families that are thought to be involved in innate immunity, i.e. GP2 is essential for host-microbial interaction and the initiation of bacteria-specific mucosal immune responses in the gut. GP2 binds to bacterial type I-fimbriae and is expressed on the outer membranes of M-cells located in the follicle-associated epithelium of intestinal mucosae. It can selectively bind a subset of commensal and pathogenic enterobacteria allowing to neutralize them and also to initiate the mucosal immune response to these bacteria. The formation of autoantibodies against these glycoproteins may either interfere with the innate immunity processes or it may reflect an immune response against an overwhelmed microbial challenge to the intestinal barrier. It is possible that GP2 and a bacterial component together are presented to the immune system as an antigen.

Biomarkers which can assist to define and predict disease outcome in various gastroenterological diseases associated with chronic inflammation of the gut has of importance. In recent years, much emphasis has been placed on the determination of important clinical or laboratory predictive factors. Identification of new markers (serologic and molecular), which can help clinicians in the identification of patients at risk for disease progression around the time of diagnosis, will hopefully allow choosing the most appropriate management in terms of therapy, intensity of follow-up and frequency of various investigations.

Our focus of clinical research is mainly paying special attention to two seemingly independent gastroenterological disease groups, namely **inflammatory bowel disease (IBD)** and **cirrhosis**, in which disturbance of innate immunity plays a significant role in the pathogenesis and disease course of these clinical entities. Uncontrolled uptake of antigens and proinflammatory molecules, including luminal bacteria and bacterial products from the gut lumen (bacterial translocation BT) due to the disruption of the mucosal immunity and also the gut barrier integrity is a common feature of these two heterogenous disease groups. Enhanced BT consequently results in a more profound activation of local and systemic proinflammatory processes and as a result, increases tissue damage. These changes altogether lead to disease progression and occurrence of complications in both entities in a different pattern with similar underlying pathogenetic mechanisms.

2. Review of literature

2.1 Inflammatory bowel diseases (IBD)

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract. Both Crohn's disease (CD) and ulcerative colitis (UC) are heterogeneous in their presentation and disease course.

Role of bacterial translocation in the pathogenesis of IBD

The etiology of CD is still not fully elucidated. However, research in recent years has brought new and important insights. Besides genetic and environmental factors, the luminal flora seems to be involved in the pathogenesis of chronic intestinal inflammation. In CD patients, the disruption of the mucosal immunity and also the gut barrier integrity may lead to uncontrolled uptake of antigens and proinflammatory molecules, including luminal bacteria and bacterial products from the gut lumen (bacterial translocation, BT). Enhanced BT results in a more profound activation of proinflammatory signalling cascade and as a consequence, increases tissue damage.

Significance of serologic antibodies in IBD

Enhanced serological antibody formation is a well-known feature of IBD. A wide range of anti-microbial and autoantibodies have been reported to be associated with either CD or UC as well as with complicated disease course. Serological antibodies have been reported to assist in the diagnosis, differential diagnosis, and prediction of either disease course or response to therapy in IBD. There is accumulating evidence from cross-sectional, longitudinal studies and meta-analyses that support the value of serological markers in identifying patients with complicated disease phenotype and increased risk of surgery in patients with CD. Anti-*Saccharomyces cerevisiae* antibody (ASCA) has been identified as the most accurate single marker in CD, yet. It is questionable if other markers can identify a specific subset of CD patients.

Anti-microbial antibodies are formed against different surface carbohydrate (anti-glycans) or protein antigens of various gut microbes. The first and still most relevant anti-microbial antibody is the ASCA (anti-*Saccharomyces cerevisiae* antibody). **Autoantibodies** are directed against various host proteins. Based on recent findings, their existence might also be related to enhanced microbial challenge to the gut due to a disturbed gut innate immune system and may trigger an exaggerated adaptive immune response. Furthermore, these serological antibodies may also be actively involved in the pathophysiology of inflammation in IBD.

Antiphospholipid antibodies (APLAs)

Antiphospholipid antibodies are a prothrombotic group of autoantibodies and established as the serological hallmark of antiphospholipid syndrome (APS). These antibodies comprise **anti-cardiolipin (ACA)**, **anti- β 2-Glycoprotein-I (anti- β 2-GPI)**, and **anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT)**. APLAs, however, are also found in a variety of disorders (chronic inflammatory diseases or post infectious conditions) not necessarily exhibiting prothrombotic activity. Even if non-prothrombotic, they may have certain pathogenetic roles in several diseases as well.

In IBD, available cross-sectional, mainly single-time point studies assessing different aspects of APLAs, came to discrepant conclusions regarding formation, prevalence, and stability of these antibodies. Their clinical significance, including association to thrombotic events in IBD is also unclear. Thus a comprehensive evaluation of the primary APLAs in a large prospectively followed-up IBD cohort is required.

Current advances may add a new spark to the investigation of the role of **anti- β 2-GPI antibodies** in the pathomechanism of IBD.

β 2-Glycoprotein I (β 2-GPI) belongs to the complement control protein (CCP) superfamily. The protein is predominantly synthesized in hepatocytes and circulates in blood in a high concentration. The presence of β 2-GPI mRNA has been demonstrated in different tissues. β 2-GPI is a single-chain protein composed five domains. Different domains are associated with the various functions of the protein. Domain 1 (D1) projects into the extracellular space and can interact with other plasma proteins and antibodies, while D5 is critical for binding anionic phospholipid membranes and various receptors such as toll-like receptors. Recently, an important function of β 2-GPI has been suggested in innate immunity. β 2-GPI has been found to scavenge lipopolysaccharide (LPS). There is a direct interaction between the D5 of β 2-GPI and LPS. Theoretically decreased level or impairment in the function of β 2-GPI may deteriorate the host defense against bacteria but it needs clarification.

Though autoantibody formation against the protein has been long known, the mechanism is not clearly understood. Polymorphisms of the β 2-GPI gene may be important for immunogenicity and the interaction of β 2-GPI with other proteins. In some studies, the Val/Leu247 single nucleotide polymorphism (SNP) in the coding region of D5 was associated with both a high frequency and stronger reactivity of anti- β 2-GPI antibodies. Presence of anti- β 2-GPI antibodies are serological hallmarks of antiphospholipid syndrome (APS). They are gain-of-function antibodies inducing new functions in the protein. Anti- β 2-GPI antibodies promote activation of endothelial cells, monocytes and platelets inducing proinflammatory, proadhesive and procoagulant phenotype in these cells. Evidence shows that besides the presence of antibodies, a second hit – usually an inflammatory event – is required for initiating pathogenetic events in the vasculature. One interesting feature of anti- β 2-GPI antibodies hitting platelet functions in the physiological haemostatic process is the neutralization of the inhibitory effect of β 2-GPI on von Willebrand factor (VWF)-dependent platelet agglutination and adhesion. Release of “active” VWF is essential to the formation of thrombi. Certain subset of anti- β 2-GPI antibodies seems to be related to certain clinical manifestations. Anti- β 2-GPI against D1 associated with an increased risk of both venous and arterial thromboembolic events, whereas antibodies recognizing D4/5 are present in non-thrombotic conditions. The role of this latter subset of antibodies, however, is less well characterized.

Apart from APS, anti- β 2-GPI has also been suggested to play roles in a variety of disorders, not necessarily thrombotic. In IBD, however, the true prevalence and the significance of anti- β 2-GPI remain unclear. Current advances may add a new spark to the investigation of the role of β 2-GPI and their antibodies in the pathomechanism of IBD. In APS, presence of cross-reactive epitopes on β 2-GPI and *Saccharomyces cerevisiae* has been reported. In a pilot study, *Papp et al.* demonstrated enhanced formation of anti- β 2-GPI in CD patients which was significantly associated with the presence of ASCA and also with the severe form of the disease. This raises the possibility that ASCA alone or by cross-reactivity with anti- β 2-GPI exaggerate the pathologic intestinal microvascular processes in IBD by interfering with the inhibitory effect of β 2-GPI on von Willebrand factor-dependent platelet adhesion and aggregation. Inflammation and coagulation are closely linked, interdependent processes in the microvasculature. Coagulation abnormalities at the mucosal level result in microthrombi formation, which are well-known features of CD and thought to be involved in

the disease pathogenesis and progression. Theoretically, impairment in the function of β 2-GPI due to the presence of anti- β 2-GPI may deteriorate certain innate immune functions as well. A novel function of the β 2-GPI protein with important relevance to innate immunity, is its ability to bind and scavenge LPS through a direct interaction between domain 5 (D5) of β 2-GPI and LPS.

Anti-pancreatic antibodies (PABs)

Enhanced formation of autoantibodies against acinar cells of the exocrine pancreas in patients with CD and celiac disease was reported. Presence of PABs was identified first by indirect immunofluorescence technique (IIFT) using cryosections of human pancreas in 1980s and has been used along with primate pancreas substrate as the primary diagnostic test for PAB so far. Two distinct but equally prevalent staining patterns exist: the reticulogranular and the droplet pattern. PABs are present up to 40% of the patients with CD and can be used to differentiate CD from UC. Furthermore some studies, including the one with the largest patient cohort, have suggested that presence of PABs is associated with specific disease phenotypes and their detection may be of clinical significance.

Despite early discovery of PABs, identification of autoantigens was unsuccessful for more than 20 years hindering the investigation of their role in the immunopathogenesis of CD. More recently, two independent groups have defined the pancreatic major glycoprotein GP2 of the zymogen granule membrane as the major target antigen of PABs applying different methods. In the study of *Komorowski et al.*, an equally relevant target antigen of PABs, the CUZD1 (CUB and zona pellucida-like domains 1), was also identified. Furthermore, they verified that the formerly reported reticulogranular pattern in tissue sections of human pancreas is strictly correlated with the presence of **anti-CUZD1**, whereas droplet pattern is to the presence of **anti-GP2 antibodies**. Furthermore, investigation of GP2 and CUZD1 revealed that they belong to protein families involved in innate and adaptive immunity. Presumably GP2 and CUZD1 may be involved in maintaining the balance between tolerance to commensal bacteria and immune response against pathogens.

Identification of the autoantigens has facilitated the development of new diagnostic tools for evaluation of PABs, such as enzyme-linked immunosorbent assay (ELISA) with isoform 4 of recombinant GP2 or advanced CUZD1 and GP2 expressing cell-based IIFT. Experience with these newly available techniques for PAB detection is scarce, especially on the clinical importance of PABs in CD. Recombinant GP2-based ELISA systems were used primarily in these studies. In contrast, information about anti-CUZD1 is very limited and lack of anti-CUZD1 antibody detection in these studies lead to missing more than half of PAB seroreactivity. Presence of anti-GP2 was associated to stricturing behavior with perianal disease in some but not all studies. However, studies were limited by cross-sectional design. Longitudinal follow-up studies are needed to unravel the clinical importance of these antibodies in the prediction of CD prognosis. For this, studies should also investigate long-term stability of PABs.

In addition, considerable amount of PAB antigens are released from exocrine pancreas into the intestinal lumen along with digestive enzymes, and they belong to protein families involved in innate and adaptive immunity. Presumably GP2 and CUZD1 may be involved in maintaining the balance between tolerance to commensal bacteria and immune response against pathogens. More data are available on GP2 and its presence was confirmed at the intestinal site of inflammation in patients with CD. GP2 is mainly present in the intestinal tract and selectively binds a subset of commensal and pathogenic enterobacteria, including *Escherichia coli*, by recognizing FimH, a component of type-I-pili on the bacterial outer membrane. GP2 is expressed on the apical plasma membrane of Peyer's patch M cells

(follicle associated epithelium) and serves as a transcytotic receptor for mucosal antigens. The protein is a prerequisite for initiating the mucosal immune response to the type-I-piliated bacteria. GP2 has also been identified as the major zymogen granule membrane glycoprotein of pancreatic acinar cells. As a component of pancreatic juice, GP2 is abundantly released from the exocrine pancreas into the gut lumen and may act as a soluble receptor of type-I-piliated bacteria. The formation of autoantibodies against these glycoproteins may either interfere with the innate immunity processes or it may reflect an immune response against an overwhelmed microbial challenge to the intestinal barrier. Autoantibodies against GP2 may interfere the binding of these bacteria. Inhibition of soluble form of GP2 may results in a direct mucosal bacterial overload. It may be hypothesized that the inappropriate bacterial transcytosis in M cells due to the presence of anti-GP2 may attenuate the antigen presentation to dendritic cells and may subsequently initiate antigen-specific mucosal immunity including IgA+ B-cell formation leading to enhanced bacterial invasion into the intestinal lamina propria. This theory, however, is yet to be confirmed.

Relevance of research to IBD

Clinical presentation at diagnosis and disease course of both CD and UC are heterogeneous and variable over time. Serologic and molecular markers which can assist to define and predict disease outcome in IBD has of importance. In recent years, much emphasis has been placed on the determination of important predictive factors. Identification of new markers, which can help clinicians in the identification of patients at risk for disease progression around the time of diagnosis, will hopefully allow choosing the most appropriate management in terms of therapy, intensity of follow-up and frequency of various investigations. Complexity of CD and UC, however, is not limited to clinical manifestation, but extends to the underlying pathogenic mechanisms. Until the exact cause and mechanisms of IBD are fully understood, each pathogenetic component should be pursued in detail. In view of the fact that IBD is typically chronic in nature factors responsible for the maintenance of gut inflammation should attract special attention. The coagulation system is a dynamic participant in the multifaceted process of chronic intestinal inflammation and represents an important component of IBD pathogenesis. Only limited data are available about the intimate relationship between coagulation system and innate immunity cells have made it a promising target for research in IBD.

2.2 Cirrhosis

Clinical presentation and disease course of cirrhosis are heterogeneous and variable over time. During the evolution of the disease, acute decompensation events associated with organ failure(s) – so-called acute-on chronic liver failure (ACLF) episodes – and chronic decompensation with progression of liver fibrosis and development of disease specific complications comprise distinct clinical entities with different immunopathology mechanisms.

Immune dysfunction in cirrhosis

Cirrhosis is the final stage of chronic liver diseases due to any cause and is associated with various levels of immune dysfunction that referred to as *cirrhosis-associated immune dysfunction syndrome (CAIDS)*. Acquired alterations, of both the innate and the adaptive immune functions are diverse, encompassing recognition, effector and regulatory mechanisms. Paradoxically, depression and overstimulation exists concurrently in the system

and results in an enhanced susceptibility to acute inflammatory processes and their exaggerated courses both locally and far from the portal of entry of the microbes or the non-microbial toxic agents. The worst consequence of the imbalance in the pro- and anti-inflammatory processes is the development of acute-on-chronic liver failure (ACLF). The subtle immune dysfunction, however, also favors to shifting towards persistence of the inflammation leading to progression of liver fibrosis and development of different complications (portal hypertension and hepatic encephalopathy). From a pathogenetic point of view, the predominant mechanisms are different during acute and chronic worsening of the liver function in cirrhosis. Enhanced bacterial translocation (BT) associated with systemic endotoxemia and an increased occurrence of systemic bacterial infections has, however, substantial impact on both clinical situations. The other important feature is that the immune status of patients is not constant during the illness and the extent of the acquired immune dysfunction is related to severity and etiology of the liver disease. The more severe the liver disease, the more subtle is the immune dysfunction. In the case of alcoholic etiology more profound alterations are generally expected. Lastly, in cirrhosis, the clinical effect of inherited variations of innate immunity gene functions are more pronounced compared to non-cirrhotic cases due to a pre-existing acquired immune dysfunction with limited compensatory mechanisms.

Role of bacterial translocation in cirrhosis

Development of hepatic fibrosis represents a complex disease trait modulated through the interaction of host genetic and environmental factors. The main stage of this interplay is the gastrointestinal tract, especially the intestinal mucosa, which is one of the largest surface where the organism interacts with the outside world. Substantial involvement of the gut luminal flora has just been revealed in the pathogenesis of chronic inflammation of the liver.

Intestinal bacterial overgrowth, altered composition of gut microbiome, bowel dysmotility, impaired local intestinal mucosal immunity, and multifactorial disruption of the intestinal mucosa barrier (increased oxidative stress, mucosal oedema and consequential mucosal structural changes causing an enhanced intestinal permeability) all together results in pathologic BT in cirrhosis. Bacterial translocation (BT), or in other words, the passage of bacteria or bacterial products from the gut into the circulation, leads to sustained uncontrolled uptake of antigens, including bacteria, mainly Gram-negative ones, and bacterial products (lipopolysaccharide [LPS], unmethylated CpG containing deoxyribonucleic acid [DNA] and lipoteichoic acid [LTA]) from the gut lumen with aforementioned mechanisms. Moreover decreased capacity of the liver to filter these bacterial products by hepatic resident macrophages (Kuppfer cells [KC]) and reduced LPS scavenging capacity of albumin due to oxidization and low levels of high density lipoprotein (HDL) and apolipoprotein A-I (ApoA-1) further assist the elevation of the above mentioned, potentially immunogenic bacterial products in the *portal* and systemic circulation.

Enhanced BT results in a more profound activation of proinflammatory signaling cascade and as a consequence, increases tissue damage of the liver. **Hepatic stellate cells (HSCs)** are the ultimate effectors of TLR ligand-mediated fibrogenesis in the liver and that maintenance of liver homeostasis depends upon the summation of pro- and anti-fibrotic effects of various immune cells on HSCs. Sustained exposure to LPS and bacterial DNA cause HSC activation via toll-like receptor mediated signalling pathways (mainly TLR-4 and -9) resulting in a shift toward **profibrogenic processes** in the fragile balance of liver homeostasis.

Changes in TLR signalling pathways are due to the prolonged exposure to intestine-derived bacterial products (LPS, unmethylated CpG containing DNA and LTA), foreign toxic

agents (ethanol and acetaldehyde derived adducts) and also damaged hepatocyte derived endogenous TLR ligands are well-established components of CAIDS. TLR2 and TLR9 recognize their ligand, di- and triacyl lipoproteins and unmethylated CpG-DNA, respectively, while TLR4 activation is triggered by the lipid A component of LPS. Functional impairment of TLR2 and TLR4, the most important PRRs for bacterial recognition, due to **the sustained LPS exposure** appears to play a significant role in the **risk of infection** in cirrhotic patients. Changes in TLR expression in response to acute or chronic stimuli are represented in parenchymal and non-parenchymal hepatic cells, as well as peripheral blood mononuclear cells (PBMCs). Although LPS and other TLR ligands can activate different signalling pathways in various cell types (immune and non-immune) promoting a proinflammatory and profibrogenic cascade in acute circumstances, anti-inflammatory and anti-fibrogenic mechanisms are present concurrently to balance these processes and maintain liver homeostasis and immunotolerance.

The phenomenon of LPS hyporesponsiveness or **LPS tolerance** is described *in vitro* in monocytes, KCs and liver sinusoidal endothelial cells (LSEC) in response to repetitive stimulation with low dose of LPS. Functional **monocyte** deactivation, a phenomenon similar to *in vitro* LPS tolerance, is also described in patients with Child C cirrhosis and ACLF. This phenomenon is presented as “**immune paralysis**” in the literature and defined as down-regulation of HLA-DR expression on monocytes. Chronic endotoxaemia was proven as the etiologic factor of “immune paralysis” by *Lin et al.* Serum LPS levels correlated inversely with HLA-DR expression and positively with serum IL-10 levels, an anti-inflammatory cytokine. Supporting this observation, *in vitro* stimulation with LPS was able to suppress HLA-DR expression in monocytes derived from healthy volunteers in an IL-10-dependent manner. Monocytes from cirrhotic patients expressing low levels of HLA-DR showed a decreased ability for TNF- α secretion accompanied by decreased expression of inducible nitric oxide synthase (iNOS) and co-stimulatory molecules (CD40, CD86). Furthermore, reduction in HLA-DR expression (<40%) was associated with poor outcome in patients with ACLF, especially if monocytes were enabled to show improvement in HLA-DR expression. The overall prognostic power, however, remains inferior to conventional markers. 90-day mortality predicted by HLA-DR expression less than 40% resulted in a specificity of 80% and sensitivity of 59% in patients with cirrhosis. In conclusion, “immune paralysis” is characterized by dominance of anti-inflammatory (elevated serum IL-6 and IL-10 levels) and suppression of pro-inflammatory processes (decreased TNF- α levels). A similar phenomenon can be observed in case of neutrophil granulocytes indicating an etiological role of enhanced BT in “**cirrhosis associated immunological dissonance**”.

Polymorphonuclear leukocytes (PMNs) are present in a fully activated state in the peripheral blood in cirrhosis potentially due to the sustained exposure to bacterial products such endotoxin. This results in an energy depleted status of the PMN with inability to function properly (decreased chemotaxis, phagocytosis and bactericidal capacity). Removal of endotoxin *in vitro* as well as attenuation of endotoxaemia *in vivo* with probiotic treatment can restore PMN dysfunction in cirrhosis further supporting this hypothesis. Increased priming and therefore “ready to act” status of PMNs is indicated by decreased L-selectin levels, overexpression of hydrogen peroxide, and increased levels of neutrophil elastase). As a result of this preparedness to defeat bacteria and PMN activation with high resting respiratory burst activity, there is an elevation in harmful reactive oxygen species (ROS) in the circulation and the PMN’s microenvironment establishing a platform for further potential cell and tissue injury. Necessarily PMNs become energy depleted and unable to respond properly for further bacterial stimuli with phagocytosis. Impaired tuftsin activity, hyponatraemia and hyperammonaemia along with inadequate generation of superoxide anion due to deficient phospholipase C (PLC) activity all contribute to the aforementioned decrease of PMN’s

phagocytic capacity. Elevated resting oxidative burst and the decreased phagocytic capacity appeared to correlate with the rate of infections and mortality. These alterations can be restored *in vitro* with endotoxin removal or GM-CSF incubation. Analogously to other innate immune cells, dichotomy in PMN function (hyperactivity then dysfunction) manifest in different ways and contribute to the pathogenic processes in the distinct stages of the cirrhosis. Recruitment of hyperactive PMNs to the liver can contribute to fibrogenesis, while exhausted PMNs with the defect in chemoattraction, enhanced adhesion to endothelial cells, and deficient migration in later stage of cirrhosis can result a deficient influx to infected sites.

A broad defect of **B-cells** in patients with ALD and its association with the exposition to circulating antigens as a consequence of shunting, or KC abnormality, or both has been known for a long while. Based on recent literature the presence of bacterial products in the circulation plays fundamental role in driving B-cell changes in cirrhosis. Soluble factors associated with BT, such as LPS and bacterial DNA, can often be detected in cirrhotic plasma and are capable of activating B-cells *in vitro*. Stimulation of B-cells by TLR ligands can lead to polyclonal activation and Ig production. Of note, in humans TLR-2, TLR-4 and TLR-8 are expressed strongly by monocytes/macrophages, but expressed poorly by B-cells. In contrast, TLR-7 and TLR-9 are expressed mainly by B lymphocytes and plasmacytoid dendritic cells. In cirrhosis, there is an enhanced serum IgA formation, mainly in those with etiology of ALD. However, the mechanisms leading to the increase of IgA levels are not fully understood. Formerly it was attributed at least partially to a defective clearance of IgA and IgA-immune complexes through altered monocytes, Fc receptor expression, and subsequent defective Fc α receptor-triggered endocytosis. For a long while, it was hypothesized that the increase of Ig synthesis in alcoholic cirrhosis might be associated with bacterial stimulation. *Massonett et al.* found significantly enhanced absolute IgA production by TLR-9 ligand CpG-activated B-cells in alcoholic cirrhosis compared to healthy subjects, in agreement with their intrinsic ability to produce spontaneously more IgA than healthy subjects. Relative TLR9 ligand CpG-induced IgA production by purified B-cells from alcoholic cirrhotic patients was, however, less prominent, in accordance with the lower TLR-9 expression on their B-cells compared to B-cells from healthy subjects. Such down-regulation of TLR-9 expression by B-cells has been reported after *in vitro* CpG treatment, suggesting that the decrease in TLR-9 expression by B-cells from patients suffering in alcoholic cirrhosis could be due to *in vivo* priming by bacterial DNA during sustained BT. Concerning IgA production, cirrhosis has another characteristic feature, namely the increased occurrence of various IgA type antimicrobial- and autoantibodies against to gut bacterial proteins or host proteins having cross-reactive epitopes with bacterial constituents in the sera of the patients. In the development of the enhanced IgA production, not only the systemic overproduction, but also the contribution of gut mucosal compartment is very probable. Composition and extent of bacterial load in the gut have a very clear effect on IgA production. Sustained exposition to bacterial antigens during BT derived from the mucosal compartment might play a central role in the enhanced IgA class antibody formation in cirrhosis. These specific antibodies were present mostly in those patients with advanced diseases and portal hypertension.

Anti-neutrophil cytoplasmic antibodies (ANCA)

Anti-neutrophil cytoplasmic antibodies (ANCA) are a group of heterogeneous antibodies. Two basic ANCA patterns are detectable in serum samples by indirect immunofluorescence (IIF) on normal peripheral blood neutrophils: the cytoplasmic (c-ANCA) and the perinuclear (p-ANCA). On ethanol-fixed neutrophil substrates the c-ANCA pattern appears as a granular, diffuse cytoplasmic fluorescence, often with accentuated fluorescence around the nuclear lobes, while typical p-ANCA reactivity results in homogeneous rim-like staining of the

perinuclear cytoplasm in some cases combined with mild nuclear staining. A third ANCA pattern of clinical importance is the so-called atypical P-ANCA staining. Atypical P-ANCA is recognized as a broad inhomogeneous rim-like staining of the nuclear periphery often with multiple intranuclear foci. By use of the cross-linking fixative formalin, typical P-ANCA diffusely labeled the cytoplasm, that is, they converted to a C-ANCA pattern. In contrast, sera containing atypical P-ANCA produced a fine “perinuclear” labeling with multiple intranuclear fluorescent foci.

These antibodies with typical staining patterns are used to diagnose and monitor the inflammatory activity in primary small vessel vasculitides. Their target antigens are well characterized. It is proteinase-3 (PR-3) and myeloperoxidase (MPO) for most c-ANCA and p-ANCA, respectively. In vasculitis, it has been suggested that ANCA may have a pathogenic role by driving the autoimmune process.

ANCAs are also found in a variety of non-vasculitic clinical conditions, namely inflammatory bowel disease, rheumatoid arthritis, chronic autoimmune liver diseases and various infections. Atypical P-ANCA is present in the sera of 50% to 90% of patients with UC and to a lesser extent in CD (10%-30%). The prevalence of the antibody is also high in patients with AIH type I (95%) and PSC (25-90%), but is detected in about 0-25% in PBC, 30% in rheumatoid arthritis and 10-20% in systemic lupus erythematosus (SLE). p-ANCA in these diseases is different from classic p-ANCA with regards to both antigen specificity and staining pattern. For distinction, it was called atypical p-ANCA, however, various different names, like x-ANCA, pANNA or DNA-ANCA also exist in published literature. Atypical p-ANCAs directed against a variety of still ill defined (nuclear or nuclear associated cytoplasmic) neutrophil antigens. The antigen specificity of these atypical ANCAs are different from the classic C- and P-ANCAs, being localized in the nuclear periphery, in contrast to the cytoplasmic location of the classic C- and P-ANCAs. Atypical P-ANCAs are most commonly seen in patients with IBD, especially ulcerative colitis, and some autoimmune liver diseases such as autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC). Some sera with atypical ANCA reactivity are positive for antibodies to elastase, lactoferrin, cathepsin G, lysosome or bactericidal permeability-increasing protein (BPI), but since they are only detected in a few atypical P-ANCA positive sera, these antigens do not appear to be the primary targets of atypical P-ANCA reactivity. Their clinical or pathophysiological significance has not been clarified yet.

Formation of ANCA may be related to the inflammatory processes. It was suggested that the death of neutrophils involved in the inflammatory response might exceed their scavenging capacity resulting in the release of cytosolic proteins of neutrophils locally at the site of inflammation, and thereby, initiating an autoimmune response. In healthy humans, natural ANCA-k mellett nagy koncentrációjú, nagy affinitású, megváltozott epitópspecifitású és funkcionálisan is aktív patológiás ANCA-k megjelenését eredményezi. Another hypothesis is that the generation of ANCA is linked to the cross-reaction between certain bacterial proteins and host antigens. It is also possible that prolonged infections can trigger the development of ANCA by molecular mimicry. Interestingly, it has been reported that the presence of atypical p-ANCAs may reflect an abnormal immune response to intestinal microorganisms. In autoimmune liver disorders, atypical p-ANCA is directed against human β tubulin isotype-5 (TBB-5) and cross-react with the bacterial protein FtsZ. This is the consequence of that TBB-5 shares an extraordinarily high structural homology with this microbial cell division protein, which is present in almost all bacteria of the intestinal microflora. Occurrence of p-ANCAs has also been considered as a sign of immunological response to enteric bacterial antigens in other diseases. Moreover, there is lack of p-ANCAs in animal models in a germ-free environment.

Our group previously reported that the presence of anti-microbial antibodies was common in patients with cirrhosis mainly in those with advanced diseases and portal hypertension, suggesting that serological response to various microbial components might be the consequence of sustained exposure to microbial antigens. Multiple levels of immune dysfunction have been reported in patients with cirrhosis rendering them susceptible to bacterial infections, which aggravated the course of illness and associated with significant morbidity and mortality. An important feature of infections in cirrhosis is the high incidence of episodes caused by enteric organisms. Bacterial translocation (BT), or in other words, the passage of bacteria or bacterial products from the gut into the circulation, is a major mechanism in the development of these infections and has a remarkable impact on course of the underlying disease. Thus, we can hypothesize that ANCAs are also frequently present in patients with cirrhosis and their presence may be associated to the clinical course of the disease, and infectious complications caused by bacteria. At present however, there are no comprehensive data concerning ANCAs in cirrhosis and its complications.

Relevance of research to cirrhosis

Thoroughly understanding of BT (1) may help identifying patients at high risk for disease progression and developing systemic infections in cirrhosis (2) may promote the development of reliable diagnostic measures of the process and (3) may support the development of non-antibiotic based, targeted therapeutic approaches those we are really lack of up to now. Achievement of these goals will hopefully assist clinicians in everyday practical decision-making when establish treatment and care strategy for the patients suffering with end-stage liver disease surmounting complications, delaying progression and diminishing mortality.

Complexity of cirrhosis, however, is not limited to clinical manifestation, but extends to the underlying pathogenic mechanisms. Until exact mechanisms of cirrhosis are not fully understood, each pathogenic component should be pursued in detail.

2. Aims

I. The aims of the present study were to investigate in a large IBD cohort (n=458) with a prospective study design focusing on antiphospholipid (APLAs) and different target specific anti-pancreatic antibodies (PAbs):

1. the prevalence and type of APLAs and PAbs;
2. to define the agreement between the new diagnostic tools for the evaluation of different target specific PAbs;
3. the long-term stability of the antibody response;
4. associations between the presence of these antibodies and clinical phenotype of the disease or its activity;
5. to determine the predictive potential of the different APLAs and PAbs with regards to the development of disease specific complications or need for surgery in a large prospective CD cohort.

II. The aims of the present study were to investigate in a large cirrhosis cohort (n=385) with a prospective study design focusing on anti-neutrophil cytoplasmic antibodies (ANCAs):

1. the prevalence, type and pattern of ANCAs;
2. associations between the presence of ANCA and the disease severity or existence of portal hypertension;
3. ANCA jelenléte – mint bakteriális transzlokációs marker – kockázati tényező-e a májcirrhotikus betegek klinikailag jelentős bakteriális fertőzéseinek kialakulásban;
4. whether presence of ANCA – as a potential marker of bacterial translocation – is associated with the development of clinically significant bacterial infections;
5. the possible origin of cirrhosis-associated ANCA.

3. Material and methods

4.1 Patient population

Inflammatory bowel diseases

We performed a cohort study among adult CD and UC patients in one tertiary IBD referral center of Hungary (Department of Gastroenterology, Institute of Internal Medicine, University of Debrecen). In all, 458 well-characterized, unrelated, consecutive IBD patients with a complete clinical follow-up (CD: 271 [male/female: 115/156, median age at presentation: 25 years (inter quartile range [IQR], 19-33)] and UC: 187 [male/female: 86/101, median age at presentation, 33 years (IQR, 23-43)]) seen at our outpatient clinic were included between January 1, 2005 and June 1, 2010. Serum samples were obtained at enrollment from each patient and frozen at -80C until testing.

Diagnosis of IBD was based on the Lennard–Jones criteria. The disease phenotype (age at onset, duration, location, and behaviour) was determined according to the Montreal Classification. Blood samples and detailed clinical phenotypes were captured at inclusion. Clinical data were determined by thorough review of patients’ medical records, which had been collected in a uniform format. Medical records that documented the disease phenotype, presence of extraintestinal manifestations (EIM) (for example, arthritis: peripheral and axial; ocular manifestations: conjunctivitis, uveitis, iridocyclitis; skin lesions: erythema nodosum, pyoderma gangrenosum; and hepatic manifestations: primary sclerosing cholangitis [PSC]), frequency of flare-ups (frequent flare-up: >1 clinical relapse/year), medication use (e.g., steroid, immunosuppressive and/or biological use at any time), need for surgery (resection in CD and colectomy in UC), the presence of familial IBD, smoking habits, and perianal involvement were retrospectively analyzed for the period prior to the prospective follow-up. At enrolment, clinical disease activity was calculated according to the Harvey–Bradshaw Index (HBI) in CD and the partial Mayo score in UC. In this study we followed the ECCO (European Crohn’s and Colitis Organisation) guidelines and defined HBI as a state of remission and ≥ 5 as a state of active disease. In case of UC ≤ 3 was defined as a state of remission and >4 as a state of active disease. Endoscopic activity was determined according to the Simple Endoscopic Score for Crohn’s Disease (SES-CD) in CD and the endoscopic component of the Mayo score in UC. SES-CD defines endoscopic activity ≥ 3 points and inactive disease ≤ 2 in CD, meanwhile in UC state of active disease was defined as invasive partial Mayo score ≥ 1 .

Phenotypical characterization of IBD patients during prospective follow-up

CD patients were enrolled into a prospective follow-up study, where the treating IBD physicians registered laboratory data, endoscopic and imaging findings, disease activity, medical treatment, date and type of complications and surgery during regular and extraordinary outpatient follow-up visits and inpatient stays. In Hungary, a follow-up visit is usually scheduled for every 6 months at a specialized gastroenterology center (the actual interval varies between 3–6 months). The treatment algorithms, both the medical and surgical, are harmonized and followed the actual ECCO guidelines. A need for surgery and timing of the resection is a consistent multidisciplinary decision with the collaboration of the gastroenterologist, radiologist and surgeon. Collected data were transferred and stored in a database for analysis. In October 1, 2013, all patients’ charts and database were reviewed and

updated for the data points (clinical end points) mentioned above. Follow-up for a particular patient was terminated if there was no further record available. Median follow-up from diagnosis was 108 months (IQR, 65-178). In CD, complicated disease behavior was defined as the occurrence of stenosis or internal penetration. Perianal fistulizing disease was distinguished from internal penetrating disease and evaluated separately. Need for surgery was defined as CD-related abdominal surgery (resection). In UC, complicated disease behavior was defined as progression of the disease extent or need for colectomy.

The control group consisted of 100 age- and gender-matched healthy blood donors (male/female: 46/54, median age, 30 years [IQR, 21-40]). The control subjects did not have any gastrointestinal and/or liver disease and were selected from consecutive blood donors in Debrecen.

Patients with cirrhosis

Sera of 385 consecutive patients with cirrhosis of different etiologies (male/female: 206/179, age: 56.6±11.0 years) were collected at the Gastroenterology Division of the 2nd Department of Medicine (Debrecen University) between May 2006 and April 2009. Mean disease duration from the diagnosis of cirrhosis was 3.9±4.2 years among patients with cirrhosis at the time of the inclusion. Diagnosis of cirrhosis was based on clinical, biochemical, imaging, and, when available, histological data. Blood samples and clinical data, including severity of cirrhosis graded according to the Child–Pugh classification and the model for end-stage liver disease (MELD) score, presence and grade of ascites and encephalopathy were captured at inclusion.

Clinical data, including age at onset, etiology, presence of esophageal varices, previous episodes of variceal bleeding and hepatic encephalopathy, prior spontaneous bacterial peritonitis (SBP) events, co-morbidities (myocardial infarction, congestive heart failure, peripheral arterial disease, cerebrovascular disease, chronic pulmonary disease, chronic renal failure, gastrointestinal ulcer disease, diabetes mellitus, and non-metastatic and metastatic cancer, including hepatocellular carcinoma, vasculitis) and current medication were collected by in-depth review of the patients' medical charts. During the study period, indications for non-selective beta-blockers and non-absorbable antibiotics either in primary or secondary prophylaxis of variceal bleeding and SBP were considered on the recommendation of current guidelines. Indications for proton pump inhibitors (PPIs) were gastroesophageal reflux disease, erosive gastritis, peptic ulcer disease or treatment for *Helicobacter pylori* infection. Etiology of cirrhosis was alcoholic in 246 (63.9%), and non-alcoholic in 139 (36.1%) cases. This latter group composed 115 patients with hepatitis C virus (HCV)-related cirrhosis and 24 ones (2.6%) with other causes.

345 of the 385 patients were available to be enrolled into a prospective follow-up study, where we registered adverse outcomes including death or development of clinically significant bacterial infections (CSI). Data were collected during regular and extraordinary outpatient follow-up visits and inpatient stays. In Hungary, a follow-up visit is usually scheduled for every 3 months at a specialized gastroenterology center (a follow-up between 1-3 months may be scheduled if dictated by disease severity or presence of disease specific complications). Follow-up period lasted 24 months or death/lost of follow up (median follow-up: 729 days [range: 5-730]). An infectious episode was defined clinically significant if it warranted hospitalization, and patients were admitted due to deterioration of general condition or liver function, or other infection-related complications, such as variceal bleeding, hepatic encephalopathy, diuretic-resistant ascites formation and renal failure. Infectious episodes were identified by reviewing medical records, including clinical symptoms, laboratory data and imaging findings, and use and efficacy of antibiotic therapy. The following laboratory data were considered: elevation of the white blood cell count (absolute: >10.8 x 10⁹/L or relative

[in patients with leukopenia]: double of count at former visits) with an elevated neutrophil rate (>76%) and elevated serum levels of high-sensitivity C reactive protein (CRP) (>10.0 mg/L) and/or procalcitonin (PCT) (>0.15 µg/L), including microbiological culture results, where available. Autopsy records (n=77) were also assessed in cases of death. The following bacterial infections were considered based on conventional criteria: infections of skin and soft tissue, orocavital region, upper and lower respiratory tract (acute bronchitis, pneumonia), biliary tract (cholecystitis, cholangitis, liver abscess), intestinal tract (gastroenteritis), urinary tract (uncomplicated cystitis were excluded), osteomyelitis, and endocarditis. Spontaneous bacterial peritonitis was diagnosed if ascitic fluid polymorphonuclear cell (PMN) count was greater than 250/mm³, with or without positive culture, in the absence of an intra-abdominal source of infection. Bacteraemia was considered when clinical symptoms and signs of infection were present and confirmed by microbiological demonstration of the causative organism from blood culture in the absence of site-specific infection.

Disease controls: Serum samples were also obtained from patients with chronic hepatitis C (chronic HCV, n=119, male/female: 50/69, age: 54.6±11.7 years) and primary biliary cirrhosis (PBC, n=102, male/female: 4/98, age: 58.8±11.8 years) without cirrhosis as disease controls. METAVIR scoring system was used to rank liver fibrosis and necroinflammatory activity. Patients with fibrosis stage 4 (F4) were considered having cirrhosis and excluded from the disease control group. The diagnosis of PBC was based on biochemical evidence of cholestasis, serum anti-mitochondrial antibodies (AMA) and/or PBC-specific AMA-M2 positivity, compatible histology, and the exclusion of extrahepatic cholestasis. The diagnosis of chronic HCV was based on positive HCV ribonucleic acid, elevated liver function tests and compatible liver biopsy.

Healthy controls: Healthy control group consisted of 100 age- and gender-matched individuals (male/female: 45/55, age: 50.5±16.7 years) selected from consecutive blood donors in Debrecen. The control subjects did not have any known gastrointestinal or liver diseases.

4.2 Laboratory methods

Serological Analysis: Blood samples were obtained at enrollment from each patient and were frozen at -80°C until testing. All the serological assays were performed in a blinded fashion without prior knowledge of the patients' diagnosis or other clinical information.

4.2.1. Detection of anti-microbial antibodies

Anti-*Saccharomyces cerevisiae* antibodies (ASCAs) are antibodies directed primarily against a 200 kDa- phosphopeptidomannan cell wall component of the common baker's or brewers yeast' *Saccharomyces cerevisiae*, while **anti-OMP PlusTM antibodies** are against multiple bacterial proteins derived from two species of intestinal bacteria (one Gram-positive and one Gram-negative). Neither bacteria are from the phylum proteobacteria, of which *Escherichia coli* is a member.

ASCA antibody evaluation in CD patients was performed by ELISA (QUANTA LiteTM, Inova Diagnostics, San Diego, CA) according to the manufacturers' instructions. The results are presented as arbitrary units, and values above the cut-off of 25 units were considered as positive. The results were documented in absolute values and in frequency of positivity.

4.2.2. Detection of autoantibodies

Detection of antiphospholipid antibodies (APLAs)

Anti- β 2-GPI, ACA and anti-PS/PT levels in serum samples were tested using the semiquantitative QUANTA Lite™ a β 2-GPI, ACAIII and aPS/PT IgA, IgG and IgM kits (INOVA Diagnostics, San Diego, California). These enzyme-linked immunosorbent assay (ELISA) kits detect IgA, IgG and IgM antibodies against β 2-GPI, cardiolipin and the PS/PT complex in human serum. Plastic microwell plate wells are coated with purified β 2-GPI, cardiolipin or PS/PT complex. Upon incubation, serum β 2-GPI, cardiolipin and PS/PT IgA, IgG or IgM antibodies bind to β 2-GPI, cardiolipin or the PS/PT complex. Unbound protein is removed by washing, while bound antibodies are detected by human IgA, IgG or IgM horseradish peroxidase-labelled conjugate. A peroxidase substrate is then added. The presence of anti- β 2-GPI, ACA and aPS/PT antibodies is determined spectrophotometrically by measuring the signal intensity of each sample compared to a five-point calibration curve. All assays were performed according to the manufacturer's instructions and were considered positive when titers were above the manufacturer's pre-established cut-off points (for anti- β 2-GPI and ACA assays, ≥ 20 units for all the IgA, IgG and IgM, and for anti-PS/PT assays ≥ 30 units for both the IgG and IgM). In case of anti-PS/PT IgA the results are presented as OD due to lack of established calibrators. Values above the OD cut-off 0.795 were considered positive for anti-PS/PT IgA. This cut-off OD value represented the mean+2SD values of the healthy controls. The results were documented in absolute OD values and in frequency of positivity. Of the 458 IBD samples obtained at enrollment, serologic analysis was technically successful in 451 of the 458 IBD cases.

Detection of anti-pancreatic antibodies (PABs)

Detection of antibodies to GP2 by enzyme-linked immunosorbent assays (ELISA)

Glycoprotein 2 autoantibodies were detected in sera of patients and controls using two different ELISAs employing recombinant human GP2 isoform 4 as solid-phase antigen (anti-MZGP2 IgA and IgG QUANTA Lite® ELISA [Inova Diagnostics, San Diego, CA, Research Use Only] and anti-GP2 IgA and IgG ELISA [GA Generic Assays, Dahlewitz/Berlin, Germany]) according to the manufacturer instructions. Briefly, 100 μ l of pre-diluted sera (1:100) was added to separate wells of MZGP2 or GP2 antigen-coated polystyrene microwells and incubated for 30 min. and 60 min., respectively at room temperature. Unbounded sample was then washed away and peroxidase-conjugated goat anti-human IgA antibody or anti-human IgG antibody was added to each well and developed with ready-to-use hydrogen peroxide/ tetramethylbenzidine chromogenic substrate. The reaction was terminated with sulphuric acid and optical density of the samples was read at wavelength of 450/620 nm with the help of a Labsystem Multiscan MS plate reader (Thermo Scientific, Budapest, Hungary). Results expressed in arbitrary units (AU/ml), were calculated in reference to a kit provided calibrator. Serum samples showing ≥ 25 AU/ml for anti-MZGP2 and ≥ 20 AU/ml for anti-GP2, respectively were interpreted as positive.

Detection of antibodies to GP2 and CUZD1 by indirect immunofluorescence tests (IIFT)

Anti-GP2 and anti-CUZD1 IgA and IgG were detected in sera of patients and controls using cell-based IIFT (Morbus-Crohn Mosaic 1, Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany). Biochips coated with transfected HEK293 cells, expressing GP2 and CUZD1 separately, are applied as substrates and were co-incubated with pre-diluted sera (exactly 1:10, 1:100 and 1:1000) for 30 min. at room temperature. Unbounded sample was then washed away and fluorescein-labeled goat anti-human IgA or IgG antibodies were used

to visualize bound antibodies of the patients' sera. Evaluation was performed under using a Eurostar Plus microscope with Bluelight LED (Euroimmun Medizinische Labordiagnostika AG). According to findings of *Komorowski et al.* reaction with transfected HEK293 cells expressing CUZD1 entirely corresponds to reticulogranular PAb pattern (type 1) on frozen sections of the human pancreas, whereas GP2-HEK reaction does the droplet pattern (type 2).

Detection anti-neutrophil cytoplasmic antibodies (ANCA)

ANCA Indirect Immunofluorescence Assay

Detection of ANCA was performed by a semiquantitative indirect immunofluorescence (IIF) technique using both ethanol- and formalin-fixed human peripheral blood neutrophil substrates (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). Specimens were incubated at a 1:10 dilution in phosphate-buffered saline and the assays were performed according to the manufacturers' instructions. The presence of ANCAs was detected with fluorescein-labeled goat antihuman IgA and IgG antibody (EUROIMMUN Medizinische Labordiagnostika AG) separately. Examination and classification were performed under ultra violet light using EUROStar II Plus microscope (EUROIMMUN Medizinische Labordiagnostika AG) at a magnification of 400x. Interpretation of the immunofluorescence results was based on the behavior of the specimens on ethanol- and formalin-fixed slides and included the following patterns: c-ANCA, typical p-ANCA and atypical p-ANCA. Serum endpoint titers of ANCA equal to or greater than 1:10 are considered positive. To quantify the ANCA titer, dilutions (dilution factor 3.2 [square root of 10]) of each specimen were made starting at 1:10.

Characterizations of IgA type ANCAs in cirrhosis

We used the IIF ANCA testing system (ethanol-fixed granulocytes) described above but applied specific monoclonal mouse anti-human antibodies (in 1:30 dilution) against each IgA subtype in parallel (Acris Antibodies GmbH, Herford, Germany; anti human IgA₁ – clone: NI 69-11, anti human IgA₂ – clone: NI 512, anti human secretory (s) IgA – clone: NI 194-4). A secondary, FITC-labeled, polyclonal goat anti-mouse antibody (1:10 dilution; DAKO, Glostrup, Denmark) was also used to augment the fluorescence signal. To maximize the comparison potential of different subtyping assay results, we applied an image analysis based quantification method using a single dilution of the samples (1:10).

Samples were analyzed by a MicroOptix MX 300 TF microscope (West Medica Austria, Perchtoldsdorf, Austria) using 400x magnification. Fluorescence images were recorded in 32 bit “.bmp” format using a MicroOptix/Vision CAM V330 camera and the SB Video&Audio AV Grabber (West Medica). A green background slide (Chroma Technology Corp., Rockingham, VT, USA) was also captured at every experimental day and used to correct for illumination strength and inhomogeneity. The images were processed using the “ImageJ 1.46r” Software. Fluorescence intensities in the green channel – expressed in arbitrary units (A.U.) – of 20 individual granulocytes per field of view were determined after segmenting the thresholded image, and mean fluorescence intensity/cell \pm SD values were calculated. For individual sera, three mean A.U./ cell values were reported according to three IgA types (IgA₁, IgA₂ and sIgA) and used for further calculations.

As we intended to compare the rate of IgA₂-type and IgA₁-type of ANCA present in the cirrhotic samples we needed to standardize the fluorescence signal measured by the anti-IgA₁ and anti-IgA₂ antibodies. Because of this Quantum™ Simply Cellular® (QSC) microspheres (Bangs Laboratories, Inc., Fishers, IN, USA) coated with known number of anti-mouse antibodies were used to capture our mouse anti-human IgA₁ or IgA₂ antibodies

plus the goat-anti mouse FITC-labeled conjugate in the same dilutions that we applied in the IIF assays. The beads were mounted on glass slides and were analyzed under the fluorescence microscope using the same image processing system as described above. The fluorescence intensity of beads with an antigen-binding capacity (ABC) of 172,189 was 44.6 ± 8.2 for IgA₁ and 48.4 ± 10.4 for IgA₂, while the beads with an ABC of 490,505 provided a signal of 100.0 ± 12.2 for IgA₁ and 103.2 ± 9.7 for IgA₂, respectively. These differences statistically were not significant. Based on these data we concluded that an equal fluorescence intensity of IgA₁-type and IgA₂-type ANCA in our system shows an equal number of antibodies bound, and therefore, the fluorescence intensity values we obtained can be used directly for calculating the IgA₂/IgA₁ ANCA rate of our samples.

The reproducibility of the ANCA IgA₁/IgA₂/sIgA subtyping methods was measured by running five replicates of one positive sample in one experiment or in five separate determinations. Within-run coefficients of variation (CVs) for ANCA IgA₁, IgA₂ and secretory component were 8.0%, 5.6% and 10.2%, respectively, while between-run CVs were 10.4%, 14.9% and 13.2% for the same antibodies.

In the ANCA subtyping assays we were able to clearly distinguish the ANCA IgA negative and positive samples. The ANCA IgA₁, IgA₂ and sIgA fluorescence intensities were significantly higher among the ANCA IgA-positive cirrhotic patients compared to the healthy controls (IgA₁: 38.4 ± 12.4 vs. 14.4 ± 4.5 , IgA₂: 31.7 ± 5.7 vs. 14.4 ± 4.0 and sIgA: 35.1 ± 9.4 vs. 14.0 ± 3.1 l; $p < 0.001$ for all groups). Furthermore, these fluorescence intensities showed strong correlation with the ANCA IgA-titer – determined by the semiquantitative method (IgA₁: Spearman correlation coefficient, $R=0.63$ [95%CI: 0.51-0.73], IgA₂: $R=0.37$ [95%CI: 0.21-0.52] and sIgA: $R=0.55$ [95%CI: 0.41-0.66]; $p < 0.001$ for all groups). These results prove that the two – image analysis based quantitative and the above-described semiquantitative – IIF methods provide highly corresponding results.

The IgA subtype analysis was performed in the case of 142 ANCA IgA-positive, 20 IgA-negative cirrhotic patients and 20 healthy controls. The fluorescence intensity values for IgA₁, IgA₂ and sIgA did not differ significantly between the healthy controls and the disease controls (healthy controls: IgA₁= 12.8 ± 3.3 , IgA₂= 14.1 ± 4.5 , sIgA= 13.0 ± 2.7 ; disease controls: IgA₁= 16.0 ± 4.9 , $p=0.08$, IgA₂= 14.6 ± 3.4 , $p=0.98$, sIgA= 14.9 ± 3.1 , $p=0.06$), therefore, we merged these two populations as a unified control group (n=40). IgA₂/IgA₁ rate was calculated by dividing the fluorescence intensity of ANCA IgA₁ or ANCA IgA₂ by the sum of the fluorescence intensities of ANCA IgA₁ and IgA₂ in each serum sample. To define the individual positivity of each sample for the presence of secretory component (SC), a cut-off (23.4) was defined based on the mean+3SD fluorescence intensity value of the control group.

Determination of antigen specificity of ANCA IgA

The presence of anti-MPO IgA and anti-PR-3 IgA antibodies in sera previously positive for ANCA IgA in IIF test (n=162) were determined by enzyme-linked immunosorbent assays (ELISA) (QUANTA Lite™ MPO and QUANTA Lite™ PR-3 INOVA Diagnostics, San Diego, CA) according to the manufacturers' instructions. The results are presented as OD. Values above the O.D. cut-off 0.159 and 0.140 were considered positive for anti-MPO and anti-PR-3, respectively. In our laboratory, these cut-off OD values represented the mean+3SD values of the healthy controls (n=92). The results were documented in absolute O.D. values and in frequency of positivity.

4.2.3. Detection of NOD2/CARD15 SNP8, 12, 13 mutations

NOD2/CARD15 SNP8, SNP12, and SNP13 genotypes were performed previously in CD patients (n=235), but not in UC patients. NOD2/CARD15 variants were detected by

denaturing high-performance liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic Limited, UK). Sequence variation, observed in the dHPLC profile, was sequenced on both strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). All investigated polymorphisms were in Hardy–Weinberg equilibrium (data not shown).

4.3 Statistical analysis

Detailed descriptions of statistical methods used in our studies are available in our original articles. For statistical analysis, GraphPad Prism 6 (San Diego, CA) and SPSS 22.0 (SPSS Inc, Chicago, IL) programs were used. The statistical methods of this study were reviewed by Elek Dinya from Semmelweis University, Institute of Health Informatics, Development and Further Training. Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as means [standard deviation (SD)] or as medians [interquartile range (IQR)] according to their homogeneity. To evaluate differences between IBD and healthy control group, as well as within subgroups of patients with IBD, the following statistical methods were used. Categorical variables were compared with the Fisher's exact test or χ^2 -test with Yates correction, linear-by-linear association, as appropriate. Continuous variables were compared with parametric or nonparametric tests, like Student's t test, one-way analysis of variance (ANOVA), or Mann-Whitney's U test or Kruskal-Wallis H test with Post-hoc analysis (Dunn's multiple comparison test). We evaluated genotype distributions regarding NOD2/CARD15 functional genetic polymorphism with Hardy-Weinberg equilibrium test. In case of assessing associations of continuous variables (like e.g. titer of antibodies) we used Pearson or Spearman correlation tests. To quantify the agreement among different assays we used a κ test. The given κ coefficients describe statistically the concordance between the different assays. Kaplan-Meier survival curves were plotted for analyzing the association between categorical clinical variables or serological antibodies and complicated disease outcomes during follow-up with LogRank test or Cox-regression analysis in the time-dependent models. Cox-regression analysis was used to assess the association between categorical clinical, genetic, or serological variables and time to a first observed event (evaluated clinical end point). Variables with a $p < 0.1$ in univariate tests were selected for the multivariate testing. A 2-sided probability value < 0.05 was considered to be statistically significant. Associations are given as Spearman's r values, odds ratio (OR) and hazard ratio (HR) with a 95% confidence intervals (CI).

4.4 Ethical permission

The study protocol was approved by the regional and national committee for research ethics (DEOEC RKEB/IKEB 2006-2007, 2595-2007, 2735-2008, 2860-2008, 3515-2011, 3514-2011; ETT-TUKEB 321-81/2005-1018-EKU, 80/2007-EKU, 25403/2011-EKU, 26186/2011-EKU, 3880/2012/EKU (59/PI/2012)). Each patient was informed of the nature of the study and signed an informed consent form.

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5. Results

5.1 Evaluation of antiphospholipid antibodies (APLA) in IBD

Frequency of APLA Markers in IBD

ACA positivity was associated with increased risk for CD compared to the controls ($OR_{ACA}=10.18$, 95% CI: 3.12-33.24). Of the different isotypes, ACA IgA ($OR_{ACA\ IgA}=49.70$, 95% CI: 3.04-813.8, χ^2 -test with Yates correction) had the highest association to CD. ACA positivity was also significantly different between CD and UC. While the prevalence of anti-PS/PT was significantly different between CD and UC, there was not a significant difference between CD and the controls. No difference was found for the prevalence of anti- β 2-GPI in different groups.

Association between APLA positivity, and other serologic markers or NOD2/CARD15 genotypes in CD

In CD, no association was found between APLA and ASCA status of the patients. Neither ACA IgA nor IgG positivity differed significantly according to presence or absence of ASCA IgA and ASCA IgG. Similarly, the prevalence of ACA was not associated with the presence of major NOD2/CARD15 mutations. NOD2/CARD15 genotypes were available in 235 CD patients. The prevalence of any ACA was not different between patients with or without NOD2/CARD15 mutations (16.1% and 26.1%, $p=NS$, χ^2 -test with Yates correction).

Association between APLA positivity and actual clinical, laboratory or endoscopic activity of the disease

At the time of enrollment, 26.6% of CD patients and 27.8% of UC patients had active disease according to clinical activity scores. Occurrence of anti- β 2-GPI, ACA and anti-PS/PT was not different between the group of patients with active (2.8%, 29.2% and 18.1%) and inactive disease state (8.8%, 21.2% and 21.2%, respectively) signified by $HBI \geq 5$. Similarly, there was no correlation between the disease activity determined by partial Mayo score >4 and APLA status in UC (data not shown).

Furthermore, the prevalence of any ACA was similar between CD patients with C-reactive protein (CRP) level >10 mg/l and those with ≤ 10 mg/l (29.0% and 21.1%, $p=NS$, χ^2 -test with Yates correction).

A total of 87 CD patients had ileocolonoscopy at enrollment. The prevalence of any ACA was not different according to endoscopic disease activity denoted by a SES-CD cut-off value of ≥ 3 (inactive vs. active: 25.6% and 29.5%). Likewise, ACA IgA and ACA IgG level was not associated with CRP level, actual HBI or SES-CD score in patients with CD applying Spearman correlation analysis (data not shown).

20.3% of the CD patients showed frequent relapse during the follow-up. The ACA prevalence was not significantly different between patients with or without frequent relapse (ACA IgA 23.5% vs. 16.7%, ACA IgG 3.9% vs. 10.1% and ACA IgM 2.0% vs. 3.5%, $p=NS$ for all).

Lastly, we investigated the association between disease duration and both the presence and magnitude of serologic response in CD. The rate of any ACA positivity was the same in all four disease duration quartile groups (Q1: 26.9%, Q2: 22.7%, Q3: 22.4% and Q4: 23.0%, $p=NS$, χ^2 -test with Yates correction). The level of ACA IgA and IgG was also not associated with disease duration (Kruskal-Wallis test).

APLA Markers and disease progression in CD

A total of 154 (56.5%) CD patients had non-stricturing and non-penetrating disease (B1) according to Montreal classification at time of sampling. 143 patients were eligible for a prospective follow-up study. The median follow-up was 53.4 months (IQR, 38.0-79.3). Among these complication and surgery naïve patients, 20.3% (29/143) experienced a complication during follow-up (31.03% developed strictures, 44.83% internal penetration and 24.14% perianal perforation only). The median time to complication was 21.4 months (IQR, 8.1-43.1). In all, 9.1% (13/143) had to undergo CD-related abdominal surgery (resection) during the follow-up period. Two patients had surgical intervention without previous complication due to the development of colorectal cancer. In the remaining patients the reason for surgery was the occurrence of a complication (23.1% stenosis, 46.2% internal penetration, 15.4% both). The median time to surgery was 50.0 months (IQR, 30.5-54.5).

In patients classified as B1, the progression of the disease to a first event defined as stenosis, internal and/or perianal penetration or CD-related surgery was not associated to presence or absence of APLA positivity. Furthermore in Kaplan-Meier analysis the likelihood for earlier progression to a disease event was similar in patients with or without APLA positivity. Of the clinical factors, disease location and smoking were those that associated with time to development of first internal penetrating and/or stricturing complication and frequent relapses to development of perianal penetrating disease.

We also evaluated the possible role of APLA antibodies in patients with complications or surgery prior to sample procurement distinctly. A total of 117 (43.5%) CD patients had complicated disease behavior (56 B2 and 61 B3 patients) according to Montreal classification at time of sampling. 109 patients were eligible for a prospective follow-up study. Among these patients with previous complication and/or surgery, 39.4% (43/109) experienced a new event defined as internal and/or perianal penetration or CD-related surgery during follow-up (complication only: 11.6%, surgery only: 58.1% or both: 30.2%). The progression of the disease to a new event was not associated to presence or absence of APLA positivity. Furthermore in Kaplan-Meier analysis the likelihood for earlier progression to a disease event was similar in patients with or without APLA positivity (data not shown).

In UC patient group association between APLAs and clinical phenotype or progression of the disease was not evaluated due to the lack of increased prevalence of any APLAs in UC population.

APLA Markers and thromboembolic events in IBD

In total, 5.1% (23/452) of IBD patients had at least one thromboembolic event (14 CD and 9 UC patients). In CD, 18 events of VT, 1 event of PE and 3 events of AT were diagnosed. In UC, 8 events of VT and 1 events of AT were diagnosed. 7 (1.6%) patients had a TE also prior to diagnosis of IBD. 4 (1.5%) CD patients had recurrent TE. In women, pregnancy loss occurred in 6.4% (10/156) of CD and 6.9% (7/101) of UC patients.

CD patients presenting with VT were significantly older than those without (median age: 30.0 vs. 38.5 years, $p=0.003$). Of the investigated clinical factors and laboratory markers, previous VT event (RR: 23.0, 95%CI: 10.6-50.1) and factor V Leiden mutation (RR: 8.3, 95%CI: 2.2-31.3) were associated with the risk of VT events. At the same time, in patients with UC, frequent relapse was associated with higher risk of VT event (RR: 6.4, 95%CI: 1.7-24.1). However, none of the APLA markers were associated with increased risk of VT events in IBD.

We further investigated the probability for thromboembolic events as a function of positivity for a certain amount of APLAs out of the whole panel. However, neither CD and nor UC patients positive for multiple APLAs showed a higher probability for the development of thromboembolic events.

Stability of APLA Markers

In order to evaluate the stability in the APLA status (positive or negative for a respective antibody), we analyzed samples from same patients over various arbitrary time-points during the disease course. At least two serum samples were taken from a subgroup of CD patients (n=198) and UC patients (n=103). Median time between sample procurements were 13.5 months [IQR, 6.8–22.9 months] for CD and 12.1 months [IQR, 5.7–20.4 months] for UC patients. Interestingly, the anti- β 2-GPI status was very stable over time with respect to all three Ig subtypes. Only 1.5%-5.8% of either CD or UC patients had a change in their anti- β 2-GPI antibody status compared to the initial sample procurement. At the same time marked differences were found in case of ACA Ig subtypes. ACA IgM status, similar to anti- β 2-GPI, was also stable. In contrast, ACA IgG and even more ACA IgA status showed significant changes over time, mainly from negative to positive. Changes in antibody status were more remarkable in CD than UC (ACA IgA: 49.9% vs. 23.3% and ACA IgG: 21.2% vs. 5.8%). Stability data are not available for anti-PS/PT antibodies since measurements were only preformed on the first available serum samples of the patients.

After availability of tumor necrosis factor (TNF) antagonist therapy through National Health reimbursement system in 2008, 43.7% (110/252) of our patients received either infliximab or adalimumab treatment. We assessed the impact of post-enrolment anti-TNF therapy in the induction of new ACA antibody formation. 59.1% (55/93) of patients who received anti-TNF therapy and had negative findings for ACA IgA at the baseline were found to have positive results later on (negative to positive change), which was significantly higher compared to the proportion of 35.6% (36/101) found among patients who did not receive anti-TNF therapy at all (p=0.004). These ratios were 30.1% vs. 12.9% (p=0.033) for ACA IgG.

5.2 Evaluation of anti-pancreatic antibodies (PAb) in IBD

Frequency of anti-pancreatic antibodies in IBD

Significantly more, 10.2%, 12.2%, 10.2% and 20.8% of the CD patients were positive for anti-GP2 IgA/IgG, anti-MZGP2 IgA/IgG, anti-rPAg2 IgA/IgG and also for anti-CUZD1 (\approx anti-rPAg1) IgA/IgG antibodies compared to both UC and healthy controls (p<0.01 for all), respectively. In addition, significant differences were found for both IgA and IgG subtypes. In contrast, the positivity rate was not different in UC and controls.

Anti-GP2 and anti-MZGP2 antibody titers were higher in CD compared to UC. The difference of the median titers for all antibodies, except anti-MZGP2 IgA, between CD and UC was statistically significant (Mann-Whitney, anti-GP2 IgA p<0.001, anti-GP2 IgG p<0.001, anti-MZGP2 IgA p=0.12, anti-MZGP2 IgG p<0.001).

Correlation of anti-pancreatic antibodies formation and overall disease duration in Crohn's disease: Stability of anti-pancreatic antibodies in Crohn's disease

No association was detected between antibody status and clinical or endoscopic disease activity (actual HBI or SES-CD) at the time of sample procurement (data not shown). In addition, to evaluate the stability of PAb status (positive or negative for a respective antibody), we analyzed samples from same patients over various arbitrary time-points during the disease course. At least two serum samples were taken from the majority of CD patients (n=192) and re-tested for all the different PAb. Median time between sample procurements was 30.3 months [IQR, 15.3-48.7]. Interestingly, the status of different PAb was very stable over time regarding both IgA and IgG subtypes with only 5% of cases changing their PAb

status over time. Development of disease complications or surgical procedure did also not affect the antibody status of any of the PABs (data not shown).

Predictive potential of anti-pancreatic antibodies for disease outcomes in Crohn's disease

We analyzed the association of the different PAB markers with poor disease outcomes (development of internal penetrating and/or stricturing disease, perianal perforating disease and need for first and subsequent surgical resection) in time-dependent univariate models.

None of the PAB markers were able to predict the development of internal penetrating and/or stricturing complications in patients with inflammatory disease behavior (B1) (n=211).

Among patients without previous perianal complication (any behavior) (n=216), those who were positive for anti-CUZD1 IgA/IgG more likely progressed to develop perianal complications (pLogRank=0.008). The association was stronger for the IgA antibody subtype (pLogRank<0.001) and a quantitative association was also found with IgA antibody titers (pLogRank<0.001).

The sensitivity analysis performed in patients with B1-p only behaviour (n=169) after excluding patients with an initial B2/3-p phenotype yielded the same results regarding the development of perianal complications (pLogRank<0.001 for the anti-CUZD1 IgA).

In the surgery-naïve patient group (n=234), progression to first resective surgery was faster in patients positive for anti-GP2 IgA antibody (pLogRank=0.002) (Figure 2). At the same time, in postoperative setting, the presence of anti-GP2 IgA antibody was not able to predict a subsequent resective surgery in patients with prior surgery (n=109).

To further evaluate the predictive potential of the PAB markers, we used a Cox-proportional Hazard regression model adjusted to gender and clinical variables with a p value of < 0.1 in univariate time dependent models (Kaplan-Meier and Log-rank analysis). Anti-CUZD1 IgA and anti-GP2 IgA were analyzed separately. Anti-CUZD1 IgA was identified as an independent predictor for the development of perianal disease both in the primary model and also in the model in the same sensitivity analysis (data not shown). In contrast, the association between anti-GP2 and need for resective surgery was lost in the multivariate model.

Association between anti-pancreatic antibody positivity and clinical, serologic and genetic characteristics of Crohn's disease

Anti-MZGP2 IgA/IgG antibodies were more prevalent in patients with pediatric disease onset (A1: 28.6%, A2: 11.3%, A3: 5.7%, p=0.012) and anti-rPAg2 IgA/IgG antibodies (L1/3 vs. L2, 13.2% vs. 4.5%, p=0.032) were more frequent in patients with ileal involvement. Penetrating disease behavior at last follow-up was associated with anti-GP2 IgA/IgG (17.9% vs. 7.3%, p=0.026), anti-MZGP2 IgA/IgG (20.2% vs. 8.9%, p=0.040) and anti-rPAg2 IgA antibodies (13.1% vs. 4.8%, p=0.040). In addition, anti-GP2 IgA/IgG (14.5% vs. 6.6%, p=0.036) was associated with need for resective surgery at maximum follow-up. Of the extraintestinal manifestations, anti-rPAg2 IgA/IgG positivity was associated with PSC (37.5% vs. 9.4%, p=0.038). In contrast, PAB directed against CUZD1 (anti-rPAg1) was associated with colonic involvement (L2/L3 vs. L1, 23.7% vs. 10.5%, p=0.041 for IgA/IgG subtype), perianal disease at maximum follow-up (P1 vs. P0, 32.6% vs. 15.0%, p=0.001 for IgA/IgG subtype) and cutaneous manifestations (23.5% vs. 10.4%, p=0.044 for IgA subtype). There was an association between the presence of IgA type PABs and ASCA antibodies. The different PAB IgA antibodies were significantly more frequent in ASCA IgA positives compared to negatives (anti-GP2 IgA: 6.7% vs. 0.0%, anti-MZGP2 IgA: 14.6% vs. 1.7%, anti-rPAg1: 17.5% vs. 4.9% and anti-rPAg2: 11.7% vs. 2.4%, p< 0.01 for all). However, PAB

IgG positivity did not differ significantly according to presence or absence of ASCA IgG. The prevalence of different PABs was also not associated with the presence or absence of major NOD2/CARD15 mutations (data not shown).

Interassay study for anti-GP2 antibodies

Altogether, the agreement among the three different assays ranged from 94.0% to 95.6% for anti-GP2 IgG and from 91.6% to 96.4% for anti-GP2 IgA. The κ coefficients suggested good concordance between the different assays.

5.3 Evaluation of anti-neutrophil cytoplasmic antibodies (ANCA) in cirrhosis

ANCA in patients with chronic liver diseases

The rates of total ANCA seropositivity, including ANCA IgA and/or IgG positivity, were significantly higher in all of the CLDs as compared to healthy controls. Among CLDs, the frequency of total ANCA seropositivity was significantly higher in patients with cirrhosis compared to those without cirrhosis ($OR_{ANCA_{Total}}$ 1.96, 95% CI: 1.28–3.00, $p < 0.01$ for chronic HCV and $OR_{ANCA_{Total}}$: 4.37, 95% CI: 2.76–6.90, $p < 0.001$ for PBC). IgA class ANCA was detected significantly more frequently in patients with cirrhosis (52.2%) compared to patients with chronic HCV (10.9%) or PBC (27.4%, $p < 0.001$, for both). However, IgG class ANCA occurred more often in patients with chronic HCV (52.1%) than cirrhosis (36.4%, $p < 0.01$) or PBC (14.7%, $p < 0.001$). None of the patients with HCV had ANCA associated vasculitis.

Fluorescence staining patterns of ANCA isotypes differed among CLD groups as well. In patients with cirrhosis the c-ANCA staining emerged as the predominant fluorescence pattern of IgA class ANCA (46.3%) and atypical p-ANCA of IgG class ANCA (51.1%). The other two staining patterns distributed equally (atypical p-ANCA: 22.4% and p-ANCA: 31.3% for IgA class ANCA; p-ANCA: 27.3% and c-ANCA: 21.6% for IgG class ANCA). In contrast, the majority of ANCA patterns – for both IgA and IgG class ANCA – were atypical p-ANCA in patients with chronic HCV (84.6% and 82.5%) and PBC (82.5% and 86.7%). 15.6%, 5.0% and 3.9% of ANCA positive sera from patients with cirrhosis, chronic HCV and PBC contained simultaneously both IgA and IgG class ANCA, respectively. The concordance between ANCA patterns of different Ig classes varied in double positive sera. Complete concordance was found in chronic HCV (100.0%) but in PBC and in cirrhosis only 50.0% and 35.0% of sera showed the same ANCA pattern using anti-IgA and IgG secondary antibodies respectively.

Characterizations of IgA type ANCAs in cirrhosis

In order to characterize the ANCA IgA antibodies in cirrhosis, ANCA IgA positive samples in the semi quantitative IIF assay were evaluated further. Proportion of IgA subclasses and presence of SC of each tested samples were measured simultaneously. The proportion of ANCA IgA₂ subtype was markedly elevated: $46.2 \pm 8.5\%$ and was only slightly lowered than ANCA IgA₁ subtype ($53.8 \pm 8.5\%$). The distribution of IgA₂ proportion was similar among the three different ANCA patterns (c-ANCA: $45.8 \pm 8.1\%$, p-ANCA: $48.0 \pm 6.8\%$ and atypical p-ANCA: $44.9 \pm 8.6\%$). The presence of SC was high, 86.8% of ANCA IgA positive samples showed positivity for this component.

We also evaluated the antigen specificity of ANCA IgA positive sera for MPO and PR-3. The frequency of anti-PR3 and anti-MPO positivity was low. 4.9% of p-ANCA positive and 16.5% of c-ANCA positive samples showed anti-MPO and anti-PR3 positivity,

respectively.

Association between ANCA and severity of disease, presence of disease specific complications or anti-microbial serology response in patients with cirrhosis

In patients with cirrhosis, ANCA IgA positivity (cases with IgA+/IgG- or IgA+/IgG+) increased gradually according to disease severity as rated by the Child-Pugh stage. Presence of ANCA IgA (cases with IgA+/IgG- or IgA+/IgG+) was also positively ($OR_{ANCAIgA}$: 3.0, 95%CI: 1.90-4.71) associated with the presence of ascites. Similarly to seropositivity rates, the more severe the disease, the higher were the titers of the ANCA IgA. ANCA IgA titers were also significantly higher in patients with ascites as compared to those ones without.

These associations were also verified if we re-analyzed them for alcoholic and non-alcoholic subgroups separately. Some etiological differences in the ANCA IgA response, however, were revealed. More enhanced ANCA IgA formation was found in alcoholic patients as compared to non-alcoholic ones. ANCA IgA positivity rates were significantly higher (by 20%) in alcoholic patients in each severity group as compared to rates of corresponding severity group in non-alcoholic patients. Furthermore, alcoholic patients with the least severe disease (Child A or no ascites) have already showed marked ANCA IgA positivity rate (53.4% or 55.9%).

On the contrary, ANCA IgG response behaved oppositely to ANCA IgA in non-alcoholic patients. Presence of ANCA IgG only (cases with IgA-/IgG+) was decreased gradually according to disease severity as rated by Child-Pugh score and negatively associated with the presence of ascites ($OR_{ANCAIgGonly}$: 0.28, 95%CI: 0.12-0.66). In alcoholic patients, however, the positivity rate for ANCA IgG only (cases with IgA-/IgG+) was low independently of diseases severity or presence of ascites. Alcoholic patients with the least severe disease (Child A or no ascites) have already showed markedly decreased ANCA IgG only positivity rates (15.5% or 13.5%) and these were significantly lower than the rates of patients with non-alcoholic disease with the same severity (41.9% or 43.0%, $p<0.001$ for both). These differences were ceased in Child C group.

ASCA IgA and anti-OMP^{Plus} IgA antibodies were more prevalent in patients with ANCA IgA positivity as compared to those without (59.0% vs. 23.4%, $p<0.001$ and 75.5% vs. 50.5%, $p<0.001$). Similar association was found between the presence of ASCA IgG and ANCA IgG antibodies. The presence of ASCA IgG was 23.6% in ANCA IgG positive and 11.5% in ANCA IgG negative patient group ($p<0.01$).

Bacterial infections: general characteristics

A total of 187 clinically significant infectious episodes were identified in the 345 patients with cirrhosis during the 2-year-long follow-up. 110 patients (31.9%) developed some type of clinically significant infections of which 39.4% suffered more than one episode. The distribution of different severe infections was as the following: 27.5% spontaneous bacterial peritonitis, 17.5% pneumonia, 12.5% urinary tract infection, 8.9% skin and soft tissue infections and 16.5% miscellaneous. The origin of the infection could not be identified in 17.1% of the cases. Bacteria were Gram-negative in 60.9% and Gram-positive in 39.1% of positive cases. The proportions of different types of infection regarding either their location or Gram specificity were similar among patients with or without ANCA (data not shown).

In the study population, the clinical factors known to affect risk of infections were also evaluated. Eighty-four (24.3%) patients had advanced disease (Child C) and 158 (45.8%) had ascites. Among patients with ascites, 36 (22.8%) had prior SBP episode of whom 14 (38.9%) suffered more than one episode. Patients with history of prior episodes of variceal bleeding or

hepatic encephalopathy were 76 (22.0%) and 57 (16.5%), respectively at the time of entry to the follow-up study. One hundred and eighty-three patients (53.0%) got non-selective beta-blockers, which rate was significantly higher in ANCA IgA positive patients as compared to ANCA IgA negative ones (60.0% vs. 45.6%, $p < 0.01$). This corresponds to the finding that ANCA IgA positive patients had more advanced disease. Use of proton pump inhibitors was 38.2% and distributed equally among ANCA IgA positive and negative patients (37.4% vs. 39.1%).

Clinical and laboratory predictors of clinically significant bacterial infection

Of the clinical factors, the disease severity according to the Child–Pugh stage ($p < 0.001$), presence of ascites (OR: 3.02; 95% CI: 1.89–4.84, $p < 0.001$), history of hepatic encephalopathy (OR: 2.15; 95% CI: 1.20–3.86, $p < 0.01$) and co-morbidities (OR: 2.03; 95% CI: 1.28–3.22, $p < 0.01$) were identified as risk factors for the development of CSI in univariate analysis (χ^2 -test or χ^2 -test with Yates correction). History of prior SBP episode was a risk factor for the development of further SBP episode (OR: 2.87; 95% CI: 1.20–6.89, $p < 0.01$).

Patients with ANCA isotype IgA presented with infectious episodes significantly more frequently compared to patients without ANCA isotype IgA (38.9% vs. 24.1%, $p < 0.01$ at the positivity cut-off titer 1:10 and 41.8% vs. 23.4%, $p < 0.001$ at titer 1:32). The infection rate was associated to the magnitude of the ANCA IgA serologic response (negative: 24.1%, titer of 1:10: 18.2%, 1:32: 42.9% and $\geq 1:100$: 54.7%, $p < 0.001$). The highest infection rate was observed in ANCA IgA positive patients with titer of 1:100 or higher (≥ 100). In this subgroup the occurrence of CSI was 54.7%. 51.1% of the ANCA IgA positive patients (90/176) belonged to this subgroup. It was 26.1% (90/345) of the whole cohort including ANCA IgA negative patients as well. Similarly, ANCA IgA patterns were associated to the development of CSI as well. Of the different ANCA IgA types, the presence of c-ANCA pattern was associated with the highest risk for the CSI (OR: 2.71; 95% CI: 1.54–4.75, $p < 0.001$). Of note, 80.2% of sera with c-ANCA pattern showed an ANCA IgA titer ≥ 100 compared to those with non-c-ANCA pattern (25.8%, $p < 0.001$).

Clinical and laboratory parameters associated with time to first clinically significant bacterial infection

In a Kaplan-Meier analysis, Child-Pugh stages, presence of ascites, co-morbidities, history of hepatic encephalopathy ($p < 0.01$) and variceal bleeding ($p = 0.046$) were associated to time to first CSI. In addition, history of prior SBP episode were associated to time to further SBP episode ($p < 0.01$).

A shorter time to first infection was found for patients with ANCA IgA, compared those without infection during the 2-year follow-up period (HR: 1.83 95%CI: 1.26-2.66, $p < 0.01$ at the positivity cut-off titer 1:10 and HR: 2.15 95%CI: 1.46-3.15, $p < 0.001$ at the positivity cut-off titer 1:32). Of the different ANCA IgA types, the presence of c-ANCA pattern was associated with the highest risk for the CSI (HR: 3.04 95%CI: 1.83-5.04, $p < 0.001$).

A Cox-regression model was also used to investigate the influence of ANCA IgA positivity on the development of CSI. After adjusting for gender, co-morbidities and disease severity according to Child–Pugh stage, ANCA IgA positivity at the cut-off titer 1:32 was independent variable associated with shorter time to first infection ($p = 0.006$). Our choice for using Child-Pugh stage to adjust the disease severity in our multivariate model was the fact that it is the best-known variable bearing significant impact on the development of bacterial

infection in cirrhosis and encompasses parameters indicating parenchymal insufficiency and portal hypertension in the most complex way. To avoid redundancy in our multivariable model, individual components or related factors to Child-Pugh score were not involved in parallel even if they were significantly associated to the development of CSI in univariate analysis.

Survival analysis

In total, 77 patients (22.3%) died during the 2-year follow-up. Kaplan-Meier survival analysis demonstrated a significantly worse survival in patients with advanced disease according to Child-Pugh stage ($p<0.001$), ascites ($p<0.001$) or co-morbidity ($p<0.01$). The presence of CSI ($p<0.001$) but not the ANCA IgA positivity ($p=0.117$) was associated to a significantly higher mortality rate.

6. Major scientific contributions

- 1.1 To our knowledge, this is the largest study to investigate prospectively the prevalence, type, and clinical significance of multiple APLAs simultaneously in a cohort of IBD patients to date.
- 1.2 Three different antibodies were assessed by ELISA. Contrary to routine laboratory practice, APLAs were identified by anti-IgA secondary antibody in addition to anti-IgG and anti-IgM isotypes.
- 1.3 Moreover, in the present study we also provided an overview of relevant APLA studies in IBD.
- 1.4 A clear strength of our study was prospective follow-up design and the application of the widest panel of currently available APLAs.
- 1.5 We demonstrated, for the first time, that enhanced ACA IgA formation is a feature of CD; the presence of ACA IgA was significantly higher as compared to UC and HC.
- 1.6 At the same time, we did not find any association between the presence of APLA and other well known prognostic serologic (ASCA IgA or IgG status of patients) or genetic markers (NOD2/CARD15 genotype).
- 1.7 In the present study we also extensively evaluated simultaneously the relationship between three disease activity parameters (clinical, laboratory and endoscopic) and APLA formation, but found no significant association.
- 1.8 Neither the presence of ACA, nor the titers of the antibodies were not associated with the CD phenotype of frequent relapse. We investigated the association between disease duration and both the presence and magnitude of serologic response in CD. The level of ACA IgA and IgG was also not associated to disease duration.
- 1.9 At the same time, we did not find any association between the presence of ACA and ASCA, even when assessing according to separate isotypes. Based on literature findings, the role of bacterial translocation (BT) in the induction of ACA, similar to anti-microbial antibodies, seems plausible. However our previously described finding implies mechanisms other than BT in the formation of ACA.
- 1.10 In the present study to enhance the potential clinical value of these markers, we applied a prospective study design, which enabled us to evaluate the potential predictive capabilities of APLAs in respect to complicated CD behavior and surgery. However, APLA did not proved as a predictive marker of the complicated disease course (stricturing, internal or perianal penetrating disease) and/or CD-associated surgery in neither clinically setting of CD.
- 1.11 Due to low frequency of different thromboembolic complications, we assessed the occurrence of these events even from the diagnosis involving the period prior to sample procurement as well. Development of VT, AT and pregnancy loss, however, did not vary according to APLA status. Of the investigated clinical factors and laboratory markers, in CD patients previous VT event and factor V Leiden mutation were associated with the risk of VT events. At the same time, in patients with UC, frequent relapse was associated with higher risk of VT event.
- 1.12 Interestingly, the anti- β 2-GPI status was very stable over time with respect to all three Ig subtypes. At the same time marked differences were found in case of ACA Ig subtypes. ACA IgM status, similar to anti- β 2-GPI, was also stable. In contrast, ACA IgG and even more ACA IgA status showed significant changes over time, mainly from negative to positive. Changes in antibody status were more remarkable in CD than UC. We assessed the impact of post-enrolment anti-TNF therapy in the induction of new ACA antibody formation. Interestingly, the patients initially negative for ACA developed ACA IgA or

IgG positivity significantly more frequently if they received anti-TNF therapy suggesting a causative association.

- 1.13 Based on our large scale and extensive evaluation of APLAs - despite their enhanced formation in IBD - they were not proved as a predictive marker of the complicated disease course in neither clinically settings of CD. Thromboembolic events were also not associated to any individual or multiple APLA positivity. Therefore use of APLAs in everyday practice and decision making cannot be recommended and do not need further study unless a newer aspect comes in future.
- 2.1 In the present study, we investigated the clinical importance of different target specific PABs in the prediction of complicated diseases behavior and surgery in adult CD patients. To our knowledge, this is the first prospective study on the new PABs.
- 2.2 For the long-term predictive potential of serologic markers it is necessary to assess the stability of antibody status over time. In terms of anti-GP2 antibody stability, only limited and conflicting data are available. In the present study long-term stability of different PABs was assessed extensively. PAB status was not associated with actual disease activity and positivity rates were stable over time in IBD patients.
- 2.3 We confirmed good agreement among newly available diagnostic tools for evaluation of anti-GP2 antibodies. Differences between the clinical phenotypes can be explained by that each test recognized also an additional patient population that was missed by the other(s), suggesting partly non-overlapping epitopes.
- 2.4 Significantly more CD patients were positive for different PABs compared to both UC patients and healthy controls. The formation of autoantibodies against GP2 and CUZD1 glycoproteins may reflect an immune response against an overwhelmed microbial challenge to the intestinal barrier. Anti-GP2 antibody titers were higher in CD compared to UC as well. The magnitude of antibody responses (titers) may correlate with the extent of bacterial translocation (BT), which is more pronounced in CD compared to UC.
- 2.5 Clinical associations were different for anti-GP2 and anti-CUZD1 antibodies in the present cohort. Prevalence of anti-GP2 antibodies was higher in our patients with pediatric onset, in extensive disease with ileal involvement, or in penetrating disease. Anti-CUZD1 antibodies were more frequent in patients with colonic involvement. Thus far, no studies have evaluated associations between target specific PABs and extraintestinal manifestations. We found that PSC was associated with the presence of anti-GP2 while cutaneous manifestations were associated with anti-CUZD1 antibodies.
- 2.6 Our findings support that PABs may contribute to better stratification of CD patients. Prevalence is relatively low and so the clinical utility of PABs in the diagnosis and prediction disease course in CD may be more modest compared to ASCA which remains the most accurate single marker in CD so far. However, anti-GP2 antibody has been shown to be more specific for CD than ASCA recently and the specificity of double positive patients with CD is 100%.
- 2.7 Of note, the prevalence rate of anti-GP2 IgA/IgG in our CD patients was even lower than those ones reported previously (10.2-12.4% vs. 21.0-45.0%). Variation in the prevalence of serologic markers among studies and in different ethnic populations is however well documented. Moreover methodological differences can also contribute to these differences.
- 2.8 Both ELISAs used in our study employed recombinant human GP2 isoform 4 as solid-phase antigen corresponding to the shorter isoform (GP2b) due to better discrimination of patients with CD from those with UC.
- 2.9 Until now, only cross-sectional associative analyses are available in CD patients. In addition, the present study provides longitudinal prospective results on the predictive

potential of PABs for identifying disease specific complications and surgery requirements. PAB positivity was able to predict faster progression to complicated disease in our patient cohort. Anti-GP2 positivity was associated with the need for surgical interventions, while anti-CUZD1 predicted development of perianal complications. One of the main strengths of our study is the analysis of outcomes during a prospective follow-up period. In the final Cox-regression multivariate model, anti-CUZD1 antibody positivity was a strong independent predictor for the development of perianal complications including age at diagnosis, sex, disease location and behavior and relapse frequency as potential confounders.

- 2.10 Interestingly, in our study IgA, but not IgG PABs types were associated with complicated diseases course in CD. The gut mucosal immune system plays a central role in the IgA antibody formation and this may at least partly reflect an immune response against an overwhelmed microbial challenge. In addition, IgA type autoantibodies are considered a sign of immunological response to enteric antigens in other diseases associated with enhanced bacterial translocation. Moreover IgA type antibodies were reported to have a pivotal role in the development of disease-specific complications compared to the IgG antibody subtype.
 - 2.11 In conclusions, the findings of our prospective referral cohort study indicate that target-specific PABs may be useful markers in the stratification of CD patients and are associated with complicated disease phenotype and risk of developing perianal complications. In addition, they may be valuable additional tools as a member of serology panels for the prediction of disease course.
- 3.1 To our knowledge, this is to date the largest study to investigate the prevalence, type and clinical significance of ANCA in patients with cirrhosis of different etiology.
 - 3.2 Prevalence and characteristics of IgG class ANCA in CLD were studied extensively; in contrast data on ANCA IgA are limited. We used IIF technique and both ethanol- and formalin-fixed human neutrophil substrates for the detection of ANCA. Contrary to routine laboratory practice, ANCA was identified by anti-IgA secondary antibody in addition to anti-IgG one. Thereafter IgA type ANCAs were subtyped as well.
 - 3.3 In the present study, we demonstrated – for the first time – that enhanced ANCA IgA formation is a feature of cirrhosis regardless of its etiology and associated with the disease severity and portal hypertension as well. Within the cirrhotic group higher ANCA IgA positivity rate and enhanced titers were found in patients with alcoholic disease compared to those with non-alcoholic one.
 - 3.4 The cause of enhanced serum IgA formation in cirrhosis has not been fully understood yet. The involvement of intestinal tract, however, is very probable. Disruption of the gut-barrier integrity at both the mechanical and immunological level is a well-known feature of cirrhosis and becomes more pronounced with disease progression. Disturbed integrity of the gut barrier with the small bowel bacterial overgrowth may in turn enable a sustained local invasion of bacterial constituents from the gut lumen (BT), which then stimulates the secretory immune system and also involved in the pathogenic processes of the disease specific complications in cirrhosis. IgA has long been accepted as an important factor in mucosal immunity, supported by basic research data, too.
 - 3.5 Since the composition and extent of bacterial load in gut has a very clear effect on IgA production and enhanced BT is a special feature of cirrhosis, we hypothesized that bacterial antigens derived from mucosal compartment and their cross-reactivity with granulocyte cytosolic or granular proteins might play central role in the enhanced IgA class ANCA formation in cirrhosis.

- 3.6 In previous publications an increase in the proportion of IgA₂ subtype and the presence of the SC concurrently considered as a confirmatory evidence for the mucosal origin of the IgA secretion. Proportion of ANCA IgA₂ subtype was markedly elevated (46%) and the presence of SC was high as well (87%) in ANCA IgA positive samples of our patients with cirrhosis irrespectively of their IIF ANCA patterns.
- 3.7 ANCA IgA antibodies was not assessed directly in the different mucosal compartments or in the organs to which bacteria are translocated (e.g. liver or ascites), which is a limitation of the present study, though our serological findings in the ANCA subtyping assays highly support our hypothesis about the ANCA IgA formation.
- 3.8 Our findings in the clinical part of the study can further support the link between the bacterial infections and the induction of ANCA formation. The occurrence of ANCA IgA positivity was significantly higher in patients with history of CSI in the past as compared those without assessed retrospectively. Moreover in a 2-year follow-up study the presence of IgA class ANCA has been proved an independent risk factor for the development of upcoming CSI as well.
- 3.9 In our patient cohort, the presence of IgA class ANCA was not associated with either the overall or the infection-related mortality. Our findings suggest that ANCA IgA has no pathogenetic role in the progression (injury, fibrogenesis) of the cirrhosis or the outcome of the bacterial complications. Even so, enhanced IgA class ANCA formation is probably not just an epiphenomenon of chronic inflammation, but an indicator of underlying mechanism that ultimately leads to CSI.
- 3.10 Antigens of IgA type ANCAs are supposedly not the classical cytosolic/granular neutrophil proteins, which were reported mainly as antigens of IgG type ANCAs in vasculitis. This hypothesis is supported by the fact, that in our cohort, occurrences of anti-PR3 and anti-MPO positivity were low. Our findings that all the three classical ANCA patterns were found among IgA type ANCAs implying multiple antigen existence at the background of their formation as well.
- 3.11 In the present study, ANCA IgG response and its changes were not parallel with the ANCA IgA response. In alcoholic patients, occurrences of IgG ANCA were much lower, approximately half of the IgA ones, already in the least severe disease stage. In non-alcoholic patients the ANCA IgG positivity rate decreased gradually according to disease severity and reached this markedly reduced level compared to IgA ANCA only in advanced disease. These alterations in ANCA IgA and IgG response clearly reflect those tendencies known from vaccination studies in this patient population and presumably reflect the impaired adaptive immune system in cirrhosis, mainly in advanced stage and direct inhibitory effect of alcohol on T cell-mediated immunity.

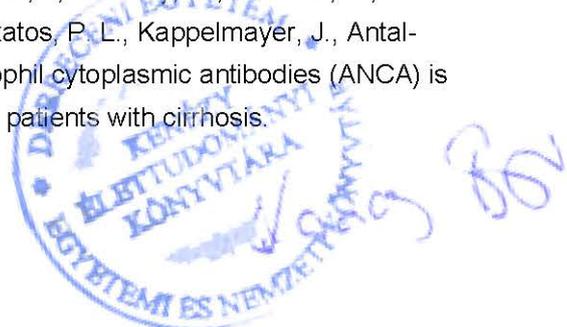


Registry number: DEENK/226/2016.PL
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Candidate: Nóra Sipeki
Neptun ID: JLUETN
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10040193

List of publications related to the dissertation

1. **Sipeki, N.**, Dávida, L., Pályu, E., Altorjay, I., Hársfalvi, J., Antal-Szalmás, P., Szabó, Z., Veres, G., Shums, Z., Norman, G. L., Lakatos, P. L., Papp, M.: Prevalence, significance and predictive value of antiphospholipid antibodies in Crohn's disease.
World J. Gastroenterol. 21 (22), 6952-6964, 2015.
DOI: <http://dx.doi.org/10.3748/wjg.v21.i22.6952>.
IF: 2.787
2. Papp, M., **Sipeki, N.**, Tornai, T., Altorjay, I., Norman, G. L., Shums, Z., Roggenbuck, D., Fechner, K., Stocker, W., Antal-Szalmás, P., Veres, G., Lakatos, P. L.: Rediscovery of the anti-pancreatic antibodies and evaluation of their prognostic value in a prospective clinical cohort of Crohn's patients: the importance of specific target antigens (GP2 and CUZD1).
J. Crohns Colitis. 9 (8), 659-668, 2015.
DOI: <http://dx.doi.org/10.1093/ecco-jcc/jjv087>
IF: 6.585
3. **Sipeki, N.**, Antal-Szalmás, P., Lakatos, P. L., Papp, M.: Immune dysfunction in cirrhosis.
World J. Gastroenterol. 20 (10), 2564-2577, 2014.
DOI: <http://dx.doi.org/10.3748/wjg.v20.i10.2564>
IF: 2.369
4. Papp, M., **Sipeki, N.**, Vitális, Z., Tornai, T., Altorjay, I., Tornai, I., Udvardy, M., Fechner, K., Jacobsen, S., Teegen, B., Sümegi, A., Veres, G., Lakatos, P. L., Kappelmayer, J., Antal-Szalmás, P.: High prevalence of IgA class anti-neutrophil cytoplasmic antibodies (ANCA) is associated with increased risk of bacterial infection in patients with cirrhosis.
J. Hepatol. 59 (3), 457-466, 2013.
DOI: <http://dx.doi.org/10.1016/j.jhep.2013.04.018>
IF: 10.401





List of other publications

5. Tornai, T., Vitális, Z., **Sipeki, N.**, Dinya, T., Tornai, D., Antal-Szalmás, P., Karányi, Z., Tornai, I., Papp, M.: Macrophage activation marker, soluble CD163 is an independent predictor of short-term mortality in patients with cirrhosis and bacterial infection.

Liver Int. [Epub ahead of print], 2016.

DOI: <http://dx.doi.org/10.1111/liv.13133>

IF: 4.47 (2015)

6. Lakatos, P. L., **Sipeki, N.**, Kovács, G., Pályu, E., Norman, G. L., Shums, Z., Golovics, P. A., Lovász, B. D., Antal-Szalmás, P., Papp, M.: Risk matrix for prediction of disease progression in a referral cohort of patients with Crohn's disease.

J. Crohns. Colitis. 9 (10), 891-898, 2015.

IF: 6.585

Total IF of journals (all publications): 33,197

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

07 September, 2016



Oral presentations

- 2013.06.15 **Sipeki N.** Az IgA típusú anti-neutrofil citoplazmatikus antitestek (ANCA) előfordulása májcirrhosisban gyakori és jelenlétük összefüggést mutat a bakteriális infekciók kialakulásával. (Laki Kálmán Doktori Iskola hallgatóinak PhD Konferenciája; Debrecen, 2013.06.15)
- 2013.11.15 **Sipeki N.**, Tornai T, Vitális Zs, Altorjay I, Tornai I, Antal-Szalmás P, Papp M. Az IgA típusú anti-neutrofil citoplazmatikus antitestek (ANCA) előfordulása májcirrhosisban gyakori és jelenlétük összefüggést mutat a bakteriális infekciók kialakulásával. (Magyar Belgyógyász Társaság Északkelet-Magyarországi Szakcsoportjának Kongresszusa, Nyíregyháza, 2013.11.15, **E-28.**)
- 2014.03.08 **Sipeki N.**, Norman GL, Lakatos PL, Papp M. Antifoszfolipid antitestek (APLA) vizsgálata gyulladásoos bélbetegségekben (IBD). (Magyar Gasztroenterológiai Társaság Colon Szekciójának Tudományos Ülése, Balatonalmádi, 2014.03.07-08.)
- 2014.06.06 **Sipeki N.** Anti-foszfolipid antitestek (APLA) vizsgálata gyulladásoos bélbetegségekben (IBD) (Laki Kálmán Doktori Iskola hallgatóinak PhD Konferenciája; Debrecen, 2014.06.06)
- 2014.08.27-30 P. Antal-Szalmás1, T. Tornai, **N. Sipeki**, D. Tornai, Zs. Vitalis, I. Tornai, Z. Szabo, R. Foldesi, A. Sumegi, P.L. Lakatos, M. Papp. Presepsin: a new biomarker in the diagnosis and prognosis of bacterial infection in cirrhosis (Magyar Laboratóriumi Diagnosztikai Társaság 57. Nagygyűlése, Nyíregyháza, 2014. augusztus 27-30. **SE 1.5**)
- 2015.02.19-21 M. Papp[#], **N. Sipeki**[#], T. Tornai, I. Földi, G.L. Norman, Z. Shums, D. Roggenbuck, P. Antal-Szalmás, G. Veres, P.L. Lakatos. Presence of anti-MZGP2 IgG and IgA antibodies assessed by 2 different ELISA assays is associated with younger age at onset, stricturing disease behaviour, need for surgery and ASCA/anti-OMP PlusTM positivity in Crohn's Disease. (Európai Crohn Colitis Társaság (ECCO) 10. Kongresszusa; Barcelona, Spanyolország, 2015.02.19-21. **DOP044**) ^{#These authors contributed equally to the work and both should be considered as first authors.}
- 2015.05.31 Kurti Z., Rutka M., Farkas K., **Sipeki N.**, Golovics P.A., Lovasz B., Vegh Z., Gecse K., Kiss L., Altorjay I., Papp M., Molnar T., Lakatos P. Accelerated treatment strategy in Inflammatory Bowel Diseases: Is it associated with a change in the disease course? (Magyar Gasztroenterológiai Társaság 57. Nagygyűlése; Siófok, 2015.05.30-06.02 **E102**)
- 2015.05.31 Papp M., **Sipeki N.**, Altorjay I., Norman G.L., Roggenbuck D., Fechner K., Antal-Szalmás P., Veres G., Lakatos P.L.. Pankréász ellenes antitestek (PAb) újrafelfedezése és prognosztikai szerepük egy prospektíven követett Crohn-beteg (CD) kohorszban: specifikus target antigének jelentősége (GP2 és CUZD1). (Magyar Gasztroenterológiai Társaság 57. Nagygyűlése; Siófok, 2015.05.30-06.02, **E138**)
- 2015.05.31 Lakatos P.L., **Sipeki N.**, Altorjay I., Norman G.L., Shums Z., Antal-Szalmás P., Veres G., Papp M. Rizikó mátrix modell a betegség progresszió előrejelzésére IBD centrumban gondozott Crohn-beteg csoportban. (Magyar Gasztroenterológiai Társaság 57. Nagygyűlése; Siófok, 2015.05.30-06.02, **E104**)
- 2015.06.05 **Sipeki N.** Pankréász ellenes antitestek (PAb) újrafelfedezése és prognosztikai szerepük egy prospektíven követett Crohn-beteg (CD) kohorszban: specifikus target antigének jelentősége (GP2 és CUZD1). (Laki Kálmán Doktori Iskola hallgatóinak PhD Konferenciája; Debrecen, 2015.06.05)
- 2016.06.04-7 G. Kovacs, **N. Sipeki**, K. Palatka, I. Altorjay, K. Fechner, G. Norman, Z. Shums, G. Veres, P.L. Lakatos, M. Papp. Szerológiai markerek jelentősége a colitis ulcerosa kórlefolyásában. (Magyar Gasztroenterológiai Társaság 58. Nagygyűlése; Siófok, 2016.06.04-06.07, **Béltbetegségek A/9**)
- 2016.10.16-19 M. Papp, T. Tornai, Gy. Kovacs, **N. Sipeki**, Zs. Vitalis, I. Tornai, K. Fechner, D. Roggenbuck, D. Tornai, G.L. Norman, Z. Shums, G. Veres, P. Orosz, B. Lombay Jr., J. Gervain, G. Par, A. Par, P. L. Lakatos, F. Szalay, P. Antal-Szalmás. Target-specific anti-pancreatic antibodies are frequent in patients with primary sclerosing cholangitis and associated with poor disease outcome. (United European Gastroenterology Week (UEG Week), 2015. 10.16-19, Bécs, **OP032**) (Session Title: 215 - Mechanisms of Primary Sclerosing Cholangitis, Session Type: Free Paper, Session Date: October 17, 2016)
- 2016.10.16-19 T. Tornai, Gy. Kovacs, **N. Sipeki**, Zs. Vitalis, I. Tornai, K. Fechner, D. Roggenbuck, D. Tornai, G.L. Norman, Z. Shums, G. Veres, P. Orosz, B. Lombay Jr., J. Gervain, G. Par, A. Par, P. L. Lakatos, F. Szalay, P. Antal-Szalmás, M. Papp. Gut barrier failure biomarkers are associated with poor disease outcome in patients with primary sclerosing cholangitis. (United European Gastroenterology Week (UEG Week), 2015. 10.16-19, Bécs, **OP033**) (Session Title: 215 - Mechanisms of Primary Sclerosing Cholangitis, Session Type: Free Paper Session, Session October 17, 2016)

Poster presentations

- 1: M Papp, **N. Sipeki**, Zs Vitalis, T Tornai, I Altorjay, I Tornai, M Udvardy, K Fechner, S Jacobsen, B Teegen, A Sumegi, G Veres, PL Lakatos, J Kappelmayer, P Antal-Szalmas. Presence of IgA class anti-neutrophil cytoplasmic antibodies (ANCA) in cirrhosis – possible hint towards the involvement of gut mucosal immune system? **Falk Symposia** 191, 2013 október 3-5 (P62).
- 2: P Antal-Szalmas, **N. Sipeki**, Zs Vitalis, T Tornai, I Altorjay, I Tornai, M Udvardy, K Fechner, S Jacobsen, B Teegen, A Sumegi, G Veres, PL Lakatos, J Kappelmayer, M Papp. Presence of IgA class anti-neutrophil cytoplasmic antibodies (ANCA) in cirrhosis – possible hint towards the involvement of gut mucosal immune system? **Magyar Immunológiai Társaság 42. Kongresszusa**, 2013. október 16-18., Pécs
- 3: P.L. Lakatos, **N. Sipeki**, T. Tornai, I. Földi, I. Altorjay, G.L. Norman, Z. Shums, G. Veres, P. Antal-Szalmas, M. Papp. Risk matrix for prediction of disease progression in a referral cohort of patients with Crohn's Disease. **Európai Crohn Colitis Társaság (ECCO) 10. Kongresszusa**; Barcelona, Spanyolország, 2015.02.19-21. (P117)
- 4: L. Kiss, K. Farkas, **N. Sipeki**, Z. Kurti, P.A. Golovics, M. Rutka, B. Lovasz, Z. Vegh, K. Gecse, I. Altorjay, M. Papp, T. Molnar, P. Lakatos. Accelerated treatment strategy in Inflammatory Bowel Diseases: Is it associated with a change in the disease course? **Európai Crohn Colitis Társaság (ECCO) 10. Kongresszusa**; Barcelona, Spanyolország, 2015.02.19-21. (P469)
- 5: M. Papp, **N. Sipeki**, T. Tornai, I. Földi, G.L. Norman, Z. Shums, D. Roggenbuck, P. Antal-Szalmas, G. Veres, P.L. Lakatos. Presence of anti-MZGP2 IgG and IgA antibodies assessed by 2 different ELISA assays is associated with younger age at onset, stricturing disease behaviour, need for surgery and ASCA/anti-OMP PlusTM positivity in Crohn's Disease. **Falk Symposium 196**; Frankfurt, Németország, 2015.03.05-08. (P72)
- 6: P.L. Lakatos, **N. Sipeki**, T. Tornai, I. Földi, G.L. Norman, Z. Shums, P. Antal-Szalmas, G. Veres, M. Papp. Risk matrix for prediction of disease progression in a referral cohort of patients with Crohn's Disease. **Falk Symposium 196**; Frankfurt, Németország, 2015.03.05-08. (P52)
- 7: T. Tornai, D. Tornai, **N. Sipeki**, I. Foldi, T. Dinya, Z. Vitalis, P. Antal-Szalmas, I. Tornai, M. Papp. Soluble CD163 (sCD163) is a marker of infection in patients with cirrhosis and acute decompensation and an independent predictor of the short-term mortality. **Európai Májkutató Társaság (EASL) 50. Kongresszusa** (50th International Liver Congress); Bécs, Ausztria, 2015.04.22-26. (P0174) – Poster of distinction
- 8: T. Tornai, D. Tornai, **N. Sipeki**, I. Foldi, T. Dinya, Z. Vitalis, P. Antal-Szalmas, I. Tornai, M. Papp. Soluble CD163 (sCD163) is a marker of infection in patients with cirrhosis and acute decompensation and an independent predictor of the short-term mortality. **Falk Symposium 197**; Lisbon, Portugal, 2015.05.08-09. (P77) – Poster of distinction
- 9: P.L. Lakatos, **N. Sipeki**, I. Altorjay, G.L. Norman, Z. Shums, G. Veres, P. Antal-Szalmas, M. Papp. Risk matrix for prediction of disease progression in a referral cohort of patients with Crohn's Disease. **Digestive Disease Week 2015**; Washington, DC, USA, 2015.05.16-19. (Su1271 – Session – IBD: Disease Complications)
- 10: P.A. Golovics, K. Farkas, **N. Sipeki**, Z. Kurti, M. Rutka, L. Kiss, B. Lovasz, Z. Vegh, K. Gecse, I. Altorjay, M. Papp, T. Molnar, P. Lakatos. Accelerated treatment strategy in Inflammatory Bowel Diseases: Is it associated with a change in the disease course? **Digestive Disease Week 2015**; Washington, DC, USA, 2015.05.16-19. (Sa1156 – Session – IBD: Clinical Outcomes)
- 11: M. Papp, **N. Sipeki**, G.L. Norman, Z. Shums, D. Roggenbuck, P. Antal-Szalmas, G. Veres, P.L. Lakatos. Presence of anti-MZGP2 IgG and IgA antibodies assessed by 2 different ELISA assays is associated with younger age at onset, stricturing disease behaviour, need for surgery and ASCA/anti-OMP PlusTM positivity in Crohn's Disease. **Digestive Disease Week 2015**; Washington, DC, USA, 2015.05.16-19. (Su1272 – Session – IBD: Disease Complications)
- 12: M. Papp, **N. Sipeki**, G.L. Norman, Z. Shums, D. Roggenbuck, P. Antal-Szalmas, G. Veres, P.L. Lakatos. Rediscovery of the anti-pancreatic antibodies and evaluation of their prognostic value in a prospective clinical cohort of Crohn's patients: the importance of specific target antigens (GP2 and CUZD1). **United European Gastroenterology Week (UEG Week)**, Barcelona, 2015. 10.24-28. (P0956) (IBD II: Diagnosis and monitoring, Session Date: October 27, 2015)

13: P.L. Lakatos, **N. Sipeki**, G.L. Norman, Z.Shums, P. Antal-Szalmas, G. Veres, M. Papp. Risk matrix for prediction of disease progression in a referral cohort of patients with Crohn's disease. **United European Gastroenterology Week (UEG Week)**, Barcelona, 2015. 10.24-28. (P0957) (Session IBD II: Diagnosis and monitoring, Session Date: October 27, 2015)

14: G. Kovacs, **N. Sipeki**, K. Palatka, I. Altorjay, K. Fechner, G.L. Norman, Z. Shums, G. Veres, P.L. Lakatos, M. Papp. Significance of serological markers in the disease course of ulcerative colitis. (A-1446) **Európai Crohn Colitis Társaság (ECCO) 10. Kongresszusa; Amsterdam, Hollandia, 2016.03.16–19.** (P123) (Session Title: Clinical: Diagnosis and outcome, Session Date: March 16-19, 2016).

15: Gy. Kovacs, **N. Sipeki**, K. Fechner, G. L. Norman, Z. Shums, P. Antal-Szalmas, P.L. Lakatos, P.L. Lakatos, P. Antal-Szalmas, M. Papp. Significance of serological markers in the disease course of ulcerative colitis. **Digestive Disease Week (DDW); San Diego, CA, USA, 2016. 05.21-24.** (Su1836) (Session Title: IBD: Disease Complications, Session Date: May 22, 2016).

16: Gy. Kovacs, **N. Sipeki**, K. Fechner, G. L. Norman, Z. Shums, P. Antal-Szalmas, P.L. Lakatos, P.L. Lakatos, P. Antal-Szalmas, M. Papp. Significance of serological markers in the disease course of ulcerative colitis. **United European Gastroenterology Week (UEG Week)**; Bécs, 2016. 10.16-19 (P0291) (Session Title: IBD I., Session Date: October 17, 2016)